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TITLE OF THE INVENTION (500 characters max)

Materials and Methods for Synthesis of a Flavor and Aroma Volatile in Plants

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Date October 3, 2003

Respectfully submitted,

SIGNATURE

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REGISTRATION NO. 38,261 *(if appropriate)*

Docket Number: UF-386P

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Provisional Application
Docket No. UF-386P

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants : Harry J. Klee, Denise Tieman

Docket No. : UF-386P

For : Materials and Methods for Synthesis of a Flavor and Aroma Volatile in Plants

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DESCRIPTION

MATERIALS AND METHODS FOR SYNTHESIS OF A FLAVOR AND AROMA VOLATILE IN PLANTS

This invention was made with government support under National Science Foundation grant number 7223426-12. The government has certain rights in the invention.

Background of the Invention

Fruits are major components of the human diet contributing a large portion of vitamins, minerals, antioxidants, and fiber. While flavor and nutrition composition have clear and profound potential for positive human benefit, they have proven to be difficult traits to modify via either traditional breeding or transgenic approaches due to their generally complex biosynthetic and regulatory pathways. In fact, the biochemical descriptors that comprise flavor are poorly defined. What is typically perceived as flavor in many fruits is the product of a complex interaction among sugars, acids and multiple volatile secondary metabolites (Buttery *et al.*, 1988; Baldwin *et al.*, 2000). Synthesis and accumulation of these compounds is the result of coordinated activity of many genes that may also impact additional aspects of plant growth and development. Effective manipulation of these traits for human benefit will therefore require greater knowledge of the pathways involved and the regulatory systems which control them. Prior to the advent of genomics, researchers could focus on the activity of only one to several genes important in a process of interest and could view their respective effects in relative isolation. From a practical perspective, flavor and nutrition are intimately related and equally important as flavor directly impacts the choice of foods for consumption which, in turn, has positive nutritional consequences on the human diet.

Fruit-bearing crop plants are taxonomically diverse (*e.g.*, pepper, tomato, melons, apples, bananas, grapes). However, they do share common features; most, though not all, fruits are enlarged ovaries. While our knowledge of how domesticated plants came to bear fruit or the mechanisms by which they ripen is still rudimentary, more is known about these processes in tomato (*Lycopersicon esculentum*) than in any other species (see Giovannoni (2001) for review).

Furthermore, a diverse set of Near Isogenic Lines (NILs), single gene ripening mutants, and transgenic lines represent portals through which genetic regulation of fruit development and ripening can be studied (Gray *et al.*, 1994; Giovannoni *et al.*, 1999). The diversity of genetically well characterized tomato germplasm described below (Table 1) is unparalleled in other fruiting species. Nevertheless, it is important to realize that while fruit ripening is a complex sum of coordinately regulated biochemical events that vary from species to species, key regulatory components are likely to be maintained (Hobson and Grierson, 1993). For example, one group has recently identified two genes that are essential for fruit ripening, RIN and NOR (Giovannoni, 2001; Vrebalov *et al.*, 2002). Fruit-specific, ripening-induced homologues of these genes have been identified from strawberry and banana (Vrebalov *et al.*, 2002). Strawberry undergoes a very different ripening program as compared to tomato in that strawberry is non-climacteric (*i.e.* no increase in respiration or ethylene biosynthesis during ripening) and accumulates high levels of anthocyanins rather than carotenoids during fruit maturation. Further, it is anatomically a receptacle, whereas most fruits are ovaries. Banana is interesting in that while its fruit are also expanded carpels, it is a monocot. Apparently similar ripening control shared among monocots and dicots indicates that basic ripening regulation is likely conserved through evolution. In summary, these results suggest that while specific nutritional and flavor components may vary among fruit species they are likely due to regulated metabolic flux through similar pathways with similar genetic control systems. Thus, regulatory and biosynthetic genes identified in tomato will allow for modification of the same or related compounds in a wide range of agriculturally important fruit species.

Tomato has long served as a model system for plant genetics, development, physiology and fruit ripening resulting in the accumulation of substantial information regarding the biology of this economically important plant. Many experimental tools and features of tomato make it ideal for study of fruit ripening; these include extensive germplasm collections, numerous natural, induced, and transgenic mutants, routine transformation technology, a dense and expanding RFLP map, numerous cDNA and genomic libraries, a small genome, relatively short life-cycle and ease of growth and maintenance. In addition, numerous genomic tools that have and continue to be developed include: a) over 140,000 EST sequences (~30,000 non-redundant) from 23 different tomato tissues/treatments (with one-third of the ESTs derived from fruit), b)

EST expression arrays being developed and utilized (see bti.cornell.edu/CGEP/CGEP.html) and c) recent initiation of activities toward development of a tomato physical map anchored to the genetic map to facilitate gene isolation and eventual genome sequencing (Tanksley *et al.*, NSF tomato genome project, 1992). The intense research effort in tomato fruit biology has resulted in many important discoveries that have had a broad impact on the field of plant biology, including control of gene expression by antisense technology, characterization of numerous genes influencing fruit development and ripening, characterization of genes for ethylene synthesis and perception, and the recent connection of ripening regulation and ethylene response to the molecular regulation of floral development (Vrebalov *et al.*, 2002).

Fruit maturation and ripening is the summation of biochemical and physiological changes occurring at the terminal stage of development rendering the organ edible and valuable as an agricultural commodity. These changes frequently include modification of cell wall ultrastructure and texture, conversion of starch to sugars, alterations in pigment and nutrient biosynthesis/accumulation, and heightened levels of flavor and aromatic volatiles (Rhodes, 1980; Hobson and Grierson, 1993). While some ripening effects, such as carotenoid and vitamin C synthesis and accumulation, have direct impact on the nutritive value of mature fruit, others impacting flavor and texture (*e.g.*, volatiles, sugars and acids) can have an indirect impact on human nutrition via their contributions to total consumption levels. In short, "if it tastes better" consumption will increase. This is especially critical as poor food choices exert a disproportional impact on children and members of society on lower rungs of the socio-economic ladder.

Although most fruits display modifications in color, texture, flavor and nutrient composition during maturation, two major classifications of ripening, climacteric and non-climacteric, have been utilized to distinguish fruit on the basis of respiration and ethylene synthesis rates. Climacteric fruits such as tomato, avocado, banana, peaches and apples, are distinguished from non-climacteric fruits such as strawberry, grape and citrus, by their increased respiration and ethylene synthesis rates during ripening (Lelievre *et al.*, 1998). In tomato, ethylene has been shown to be necessary for the coordination and completion of ripening (Yang, 1985; Tucker and Brady, 1987; Klee *et al.*, 1991; Picton *et al.*, 1993; Lanahan *et al.*, 1994). The critical role of ethylene in coordinating climacteric ripening at the molecular level was first

observed via analysis of ethylene inducible ripening-related gene expression in tomato (Lincoln *et al.*, 1987; Maunders *et al.*, 1987). Numerous fruit development-related genes have since been isolated via differential expression patterns and biochemical function (reviewed in Gray *et al.*, 1994). The *in vivo* functions of many fruit development- and ripening-related genes have been tested via antisense repression and/or mutant complementation in tomato. As examples, polygalacturonase was shown to be necessary for ripening-related pectin depolymerization and pathogen susceptibility, yet to have little effect on fruit softening (Smith *et al.*, 1988, Giovannoni *et al.*, 1989, Kramer *et al.*, 1990). Inhibition of phytoene synthase resulted in reduced carotenoid biosynthesis and reduction in fruit and flower pigmentation (Fray and Grierson, 1993). Reduced ethylene evolution resulted in ripening inhibition of ACC synthase (ACS) and ACC oxidase (ACO) antisense lines (Oeller *et al.*, 1991; Hamilton *et al.*, 1990) while introduction of a dominant mutant allele of the *NR* ethylene receptor resulted in plants inhibited in virtually every measurable ethylene response including fruit ripening (Wilkinson *et al.*, 1995; Yen *et al.*, 1995).

Expression analysis of multiple tomato ripening-related genes indicates that a subset exhibit developmentally-controlled ethylene inducibility, *i.e.*, they are ethylene inducible only in ripening fruits. Examples include members of the ACO and ACS gene families (Theologis *et al.*, 1993; Blume and Grierson, 1997; Nakatsuka *et al.*, 1998), the *NR* ethylene receptor (Wilkinson *et al.*, 1995; Payton *et al.*, 1996; Lashbrook *et al.*, 1998) and E8 (Deikman *et al.*, 1992). Additional evidence for non-ethylene mediated ripening control comes from analysis of gene expression in ripening impaired mutants such as *rin* (*ripening-inhibitor*) and *nor* (*non-ripening*) that fail to ripen in response to exogenous ethylene yet display signs of ethylene sensitivity and signaling including induction of some ethylene-regulated genes (Yen *et al.*, 1995). These results suggest that regulatory constraints are placed on climacteric fruit maturation in addition to general ethylene biosynthesis and signaling. Such mechanisms could include fruit-specific regulation of certain subsets of ethylene regulated genes or factors that operate separate from and in addition to ethylene as seems to be the case for both the RIN (Vrebalov *et al.*, 2002) and NOR transcription factors. This is particularly interesting as a greater understanding of the relationship between ethylene, developmental, and environmental signals will likely reveal the impact of various signaling systems on pathways impacting flavor and human nutrition. Indeed numerous environmental factors such as light and temperature can dramatically influence the

degree and rate of fruit ripening with significant impacts on the accumulation of carotenoids and flavor compounds (Hobson and Grierson, 1993; Yen *et al.*, 1997).

Numerous plant metabolites can be listed when the net of "nutritive compounds" is cast. These include various antioxidants, vitamins, minerals, fiber, lipids, and amino acids, to name just a few. In addition, as noted above, one can rationally argue that modification of flavor and additional quality attributes may lead to improved health via increased fruit or vegetable consumption.

Tomato fruits are among the highest source of lycopene, β-carotene, and vitamin C (ascorbate) in the diets of humans in the US, South America, and Europe, with steadily increasing prominence in Asia and the Middle East. In addition to direct nutritive value, carotenoids in particular are metabolized to compounds that impact flavor and aroma of fruit and thus have a significant impact on resulting fresh and processed products. Genes encoding the synthetic steps from phytoene through β-carotene (Bartley *et al.*, 1994; Ronen *et al.*, 1999) are potential regulatory points for modification of carotenoid levels. Indeed, available data indicate that accumulation of lycopene is due to coordinated up-regulation of the genes preceding its synthesis and down-regulation of genes that further metabolize it during ripening (Ronen *et al.*, 1999). Numerous mutant, transgenic, RI and breeding lines that display a wide range of levels of lycopene and β-carotene are available (Table 1). While specific mutants represent some of the catalytic steps (*e.g.*, *r* = phytoene synthase and *cr* and *B* = lycopene cyclase; Hamilton *et al.*, 1990; Ronen *et al.*, 1999) others such as *hp-1* and *hp-2* represent regulators of environmental response. Antisense phytoene synthase tomato lines are greatly reduced in all of the carotenoid-derived volatiles (Baldwin *et al.*, 2000). Furthermore, transgenic and mutant lines altered in ethylene synthesis or perception display variation in carotenoid levels (Table 1).

Table 1. Tomato germplasm altered in carotenoids, flavonoids, vitamin C.

Genotype	Carotenoids	Vit.C	Volatiles	Function
*rin; ripening-inhibitor**	very low	low	NA	MADS-box protein
*nor; non-ripening**	low	low	NA	transcription factor
*Nr: Never-ripe**	low	NA	NA	ethylene receptor
hp-2; high-pigment-2	high	high	NA	DET1 (light signaling)
cr; crimson	low B, high L	high	NA	lycopene cyclase
B; Beta	high B, low L	NA	NA	lycopene cyclase
r; Phytoene Synthase	low	NA	low	phytoene synthase
*hp-1; high-pigment-1***	high	high	NA	Not cloned (light signaling)
Nr-2: Never-ripe-2	low	NA	NA	Not cloned
Gr: Green-ripe	low	NA	NA	Not cloned
t; tangerine	low	NA	NA	Not cloned
at; apricot	low	NA	NA	Not cloned
Cnr; Clear non-ripening	low	NA	NA	Not cloned
L. esculentum x *L. pennellii* Recombinant Inbreds	low-high	low-high	low-high	
ACO; ACC oxidase*	low	NA	NA	ethylene Biosynthesis
ACS; ACC synthase*	low	NA	NA	ethylene Biosynthesis
ACD; ACC deaminase*	low	NA	NA	ethylene Biosynthesis
TCTR1; tomato CTR1*	low-high	NA	NA	ethylene signaling MAPKKK

The dashed line separates mutants for which the corresponding gene has been cloned (1st tier) from those which have not (2nd tier). The last tier indicates transgenic lines altered in ethylene synthesis or response and with corresponding changes in carotenoid accumulation. Genotypes indicated with an (*) represent those for which multiple independent transgenic lines are available demonstrating a range of carotenoid accumulation levels. **Three different mutant alleles of *hp-1* each having varying degrees of effect on carotenoid and flavonoid accumulation were provided by M. Koornneef. B = β-carotene. L = lycopene. While quantitative data for vitamin C and volatiles are unknown for many of these lines (NA), their respective phenotypes suggest they are likely to be altered in one or both.

In the case of flavor volatiles, the pathways for synthesis are in many cases not well established. For example, synthesis of apocarotenoids such as β-ionone and β-damascenone is not at all understood. Only recently has an Arabidopsis enzyme, CCD1 (Related to Carotenoid Dioxygenases), that synthesizes apocarotenoids such as β-ionone *in vitro* been identified

(Schwartz *et al.*, 2001). This gene is part of a multigene family, some of which are responsible for synthesis of other apocarotenoids such as ABA (Tan *et al.*, 1997). CCD1 cleaves multiple carotenoid substrates at the 9-10 and 9'-10' bonds, potentially releasing volatiles such as β-ionone, although this has not been established *in vivo*. Similarly, several different volatiles are derived from lipid breakdown (Table 2). The likely first step in their syntheses is the action of a lipoxygenase (LOX) (Riley and Thompson, 1997; Baldwin *et al.*, 2000). Currently there are 14 different EST contigs in the tomato database putatively identified as LOX. Any LOX exhibiting correlation with the lipid-derived volatiles would be a candidate sequence for analyses. It is exactly this sort of correlative biochemical and expression approach that resulted in identification of a key enzyme in strawberry volatile synthesis (Aharoni *et al.*, 2000).

Table 2. The 16 most significant flavor volatiles of tomato

Volatile	Conc. (ppb)	Log odor units	Precursor	Odor Characteristics
cis-3-Hexenal	12,000	3.7	lipid	tomato/green
β-ionone	4	2.8	carotenoid	fruity/floral
Hexanal	3,100	2.8	lipid	green/grassy
β-Damascenone	1	2.7	carotenoid	fruity
1-Penten-3-one	520	2.7	lipid	fruity floral/green
2+3-Methylbutanal	27	2.1	ILE/LEU	musty
trans-2-Hexenal	270	1.2	lipid	green
2-Isobutylthiazole	36	1.0	LEU	tomato vine
1-nitro-2-Phenylethane	17	0.9	PHE	musty, earthy
trans-2-Heptenal	60	0.7	lipid	green
Phenylacetaldehyde	15	0.6	PHE	floral/alcohol
6-Methyl-5-hepten-2-one	130	0.4	carotenoid	fruity, floral
cis-3-Hexenol	150	0.3	lipid	green
2-Phenylethanol	1,900	0.3	PHE	nutty
3-Methylbutanol	380	0.2	LEU	earthy, musty
Methyl salicylate	48	0.08	PHE	wintergreen

Volatiles are ranked by importance based on Odor Units (concentration X humans' ability to detect). Concentrations are average values from typical commercial tomatoes. Odor characteristics were determined by a trained expert panel.

Brief Summary of the Invention

The subject invention concerns polynucleotides encoding a plant 2-phenylethanol dehydrogenase enzyme. In one embodiment, the polynucleotide encodes a tomato 2-phenylethanol dehydrogenase. The subject invention also concerns 2-phenylethanol dehydrogenase polypeptides encoded by polynucleotides of the present invention.

The subject invention also concerns methods for providing a plant with an increased flavor and aroma volatile. Plants can be transformed with a polynucleotide of the present invention. The subject invention also concerns these transformed plant cells, plant tissue, and plants and transgenic progeny thereof.

Brief Description of the Figures

Figures 1A and 1B show the results of GC profiles of volatiles collected from M82 or introgression line 8-2-1 ripe fruit. Introgression line 8-2-1 fruit have higher levels of phenylacetaldehyde and 2-phenylethanol than control M82 fruit.

Figure 2 is a graph of phenylacetaldehyde and 2-phenylethanol levels in control M82 and *L. pennellii* introgression line 8-2-1 fruit. Data are presented as % of control M82 fruit.

Figures 3A and 3B show a full-length cDNA (SEQ ID NO. 1) and amino acid sequence (SEQ ID NO. 2) of a 2-phenylethanol dehydrogenase of the present invention.

Figure 4 shows a chemical reaction for production of volatile compound 2-phenylethanol in plants. In the reaction, phenylacetaldehyde is converted to 2-phenylethanol by a 2-phenylethanol dehydrogenase.

Figures 5A, 5B, and 5C show the result of alcohol dehydrogenase activities of 2-phenylethanol dehydrogenase on phenylacetaldehyde and related substrates. Activity is determined by the disappearance of substrate and a reduction in OD (340nm). Highest activity levels are observed with phenylacetaldehyde as a subtrate.

Figures 6A and 6B show the result of GC profiles of volatiles emitted from wild-type Mitchell Diploid (MD) petunia flowers and transgenic petunia flowers expressing the tomato 2-phenylethanol dehydrogenase gene. In transgenic flowers, higher levels of 2-phenylethanol and lower levels of phenylacetaldehyde are observed as compared to wild-type flowers.

Figure 7 is a graph of the levels of 2-phenylethanol and phenylacetaldehyde emitted from petunia flowers of wild-type Mitchell Diploid (MD) and transgenic lines expressing the tomato 2-phenylethanol dehydrogenase gene.

Brief Description of the Sequences

SEQ ID NO. 1 shows a nucleotide sequence encoding a 2-phenylethanol dehydrogenase according to the present invention.

SEQ ID NO. 2 shows an amino acid sequence of a 2-phenylethanol dehydrogenase encoded by SEQ ID NO. 1 of the present invention.

SEQ ID NO. 3 shows an oligonucleotide PCR primer that can be used according to the present invention.

Detailed Disclosure of the Invention

The subject invention concerns polynucleotides encoding a plant 2-phenylethanol dehydrogenase enzyme. In one embodiment, the polynucleotide encodes a 2-phenylethanol dehydrogenase of tomato. In an exemplified embodiment, the polynucleotide encodes a tomato 2-phenylethanol dehydrogenase polypeptide having an amino acid sequence shown in SEQ ID NO. 2, or an enzymatically functional fragment or variant thereof. In a specific embodiment, the polynucleotide encoding the amino acid sequence shown in SEQ ID NO. 2 comprises the nucleotide sequence shown in SEQ ID NO. 1, or a sequence encoding an enzymatically functional fragment or variant of SEQ ID NO. 2. In one embodiment, the polynucleotide encoding the amino acid sequence shown in SEQ ID NO. 2 comprises nucleotides 172 to 1155 of the nucleotide sequence shown in SEQ ID NO. 1.

The subject invention also concerns polynucleotide expression constructs comprising a polynucleotide sequence of the present invention encoding a plant 2-phenylethanol dehydrogenase. In one embodiment, an expression construct of the invention comprises a polynucleotide sequence encoding a tomato 2-phenylethanol dehydrogenase comprising an amino acid sequence shown in SEQ ID NO. 2, or an enzymatically functional fragment or variant thereof. In a specific embodiment, the polynucleotide sequence comprises a polynucleotide sequence shown in SEQ ID NO. 1, or a sequence encoding an enzymatically

functional fragment or variant of SEQ ID NO. 2. In one embodiment, the polynucleotide encoding the amino acid sequence shown in SEQ ID NO. 2 comprises nucleotides 172 to 1155 of the nucleotide sequence shown in SEQ ID NO. 1. Expression constructs of the invention generally include regulatory elements that are functional in the intended host cell in which the expression construct is to be expressed in. Thus, a person of ordinary skill in the art can select regulatory elements for use in bacterial host cells, yeast host cells, plant host cells, insect host cells, mammalian host cells, and human host cells. Regulatory elements include promoters, transcription termination sequences, translation termination sequences, enhancers, and polyadenylation elements. As used herein, the term "expression construct" refers to a combination of nucleic acid sequences that provides for transcription of an operably linked nucleic acid sequence. As used herein, the term "operably linked" refers to a juxtaposition of the components described wherein the components are in a relationship that permits them to function in their intended manner. In general, operably linked components are in contiguous relation.

An expression construct of the invention can comprise a promoter sequence operably linked to a polynucleotide sequence encoding a 2-phenylethanol dehydrogenase of the invention. Promoters can be incorporated into a polynucleotide using standard techniques known in the art. Multiple copies of promoters or multiple promoters can be used in an expression construct of the invention. In a preferred embodiment, a promoter can be positioned about the same distance from the transcription start site as it is from the transcription start site in its natural genetic environment. Some variation in this distance is permitted without substantial decrease in promoter activity. A transcription start site is typically included in the expression construct.

If the expression construct is to be provided in or introduced into a plant cell, then plant viral promoters, such as, for example, a cauliflower mosaic virus (CaMV) 35S (including the enhanced CaMV 35S promoter (see, for example U.S. Patent No. 5,106,739)) or a CaMV 19S promoter can be used. Other promoters that can be used for expression constructs in plants include, for example, prolifera promoter, Ap3 promoter, heat shock promoters, T-DNA 1'- or 2'-promoter of *A. tumefaciens*, polygalacturonase promoter, chalcone synthase A (CHS-A) promoter from petunia, tobacco PR-1a promoter, ubiquitin promoter, actin promoter, alcA gene promoter, pin2 promoter (Xu *et al.*, 1993), maize WipI promoter, maize trpA gene promoter (U.S. Patent No. 5,625,136), maize CDPK gene promoter, and RUBISCO SSU promoter (U.S.

Patent No. 5,034,322) can also be used. Tissue-specific, for example fruit-specific promoters, such as the E8 promoter of tomato (accession number: AF515784; Good *et al.* (1994)) can be used. Fruit-specific promoters such as flower organ-specific promoters can be used with an expression construct of the present invention for expressing a polynucleotide of the invention in the flower organ of a plant. Examples of flower organ-specific promoters include any of the promoter sequences described in U.S. Patent Nos. 6,462,185; 5,639,948; and 5,589,610. Seed-specific promoters such as the promoter from a β-phaseolin gene (of kidney bean) or a glycinin gene (of soybean), and others, can also be used. Constitutive promoters (such as the CaMV, ubiquitin, actin, or NOS promoter), developmentally-regulated promoters, and inducible promoters (such as those promoters than can be induced by heat, light, hormones, or chemicals) are also contemplated for use with polynucleotide expression constructs of the invention.

For expression in animal cells, an expression construct of the invention can comprise suitable promoters that can drive transcription of the polynucleotide sequence. If the cells are mammalian cells, then promoters such as, for example, actin promoter, metallothionein promoter, NF-kappaB promoter, EGR promoter, SRE promoter, IL-2 promoter, NFAT promoter, osteocalcin promoter, SV40 early promoter and SV40 late promoter, Lck promoter, BMP5 promoter, TRP-1 promoter, murine mammary tumor virus long terminal repeat promoter, STAT promoter, or an immunoglobulin promoter can be used in the expression construct. The baculovirus polyhedrin promoter can be used with an expression construct of the invention for expression in insect cells.

For expression in prokaryotic systems, an expression construct of the invention can comprise promoters such as, for example, alkaline phosphatase promoter, tryptophan (trp) promoter, lambda P_L promoter, β-lactamase promoter, lactose promoter, phoA promoter, T3 promoter, T7 promoter, or tac promoter (de Boer *et al.*, 1983).

Promoters suitable for use with an expression construct of the invention in yeast cells include, but are not limited to, 3-phosphoglycerate kinase promoter, glyceraldehyde-3-phosphate dehydrogenase promoter, metallothionein promoter, alcohol dehydrogenase-2 promoter, and hexokinase promoter.

Expression constructs of the invention may optionally contain a transcription termination sequence, a translation termination sequence, a sequence encoding a signal peptide, and/or

enhancer elements. Transcription termination regions can typically be obtained from the 3' untranslated region of a eukaryotic or viral gene sequence. Transcription termination sequences can be positioned downstream of a coding sequence to provide for efficient termination. A signal peptide sequence is a short amino acid sequence typically present at the amino terminus of a protein that is responsible for the relocation of an operably linked mature polypeptide to a wide range of post-translational cellular destinations, ranging from a specific organelle compartment to sites of protein action and the extracellular environment. Targeting gene products to an intended cellular and/or extracellular destination through the use of an operably linked signal peptide sequence is contemplated for use with the polypeptides of the invention. Classical enhancers are cis-acting elements that increase gene transcription and can also be included in the expression construct. Classical enhancer elements are known in the art, and include, but are not limited to, the CaMV 35S enhancer element, cytomegalovirus (CMV) early promoter enhancer element, and the SV40 enhancer element. Intron-mediated enhancer elements that enhance gene expression are also known in the art. These elements must be present within the transcribed region and are orientation dependent. Examples include the maize shrunken-1 enhancer element (Clancy and Hannah, 2002).

DNA sequences which direct polyadenylation of mRNA transcribed from the expression construct can also be included in the expression construct, and include, but are not limited to, an octopine synthase or nopaline synthase signal. The expression constructs of the invention can also include a polynucleotide sequence that directs transposition of other genes, *i.e.*, a transposon.

Expression constructs can also include one or more dominant selectable marker genes, including, for example, genes encoding antibiotic resistance and/or herbicide-resistance for selecting transformed cells. Antibiotic-resistance genes can provide for resistance to one or more of the following antibiotics: hygromycin, kanamycin, bleomycin, G418, streptomycin, paromomycin, neomycin, and spectinomycin. Kanamycin resistance can be provided by neomycin phosphotransferase (NPT II). Herbicide-resistance genes can provide for resistance to phosphinothricin acetyltransferase or glyphosate. Other markers used for cell transformation screening include genes encoding β-glucuronidase (GUS), β-galactosidase, luciferase, nopaline

synthase, chloramphenicol acetyltransferase (CAT), green fluorescence protein (GFP), or enhanced GFP (Yang *et al.*, 1996).

The subject invention also concerns polynucleotide vectors comprising a polynucleotide sequence of the invention that encodes a 2-phenylethanol dehydrogenase. Unique restriction enzyme sites can be included at the 5' and 3' ends of an expression construct or polynucleotide of the invention to allow for insertion into a polynucleotide vector. As used herein, the term "vector" refers to any genetic element, including for example, plasmids, cosmids, chromosomes, phage, virus, and the like, which is capable of replication when associated with proper control elements and which can transfer polynucleotide sequences between cells. Vectors contain a nucleotide sequence that permits the vector to replicate in a selected host cell. A number of vectors are available for expression and/or cloning, and include, but are not limited to, pBR322, pUC series, M13 series, and pBLUESCRIPT vectors (Stratagene, La Jolla, CA).

Polynucleotides of the present invention can be composed of either RNA or DNA. Preferably, the polynucleotides are composed of DNA. The subject invention also encompasses those polynucleotides that are complementary in sequence to the polynucleotides disclosed herein.

Because of the degeneracy of the genetic code, a variety of different polynucleotide sequences can encode 2-phenylethanol dehydrogenase enzymes of the present invention. A table showing all possible triplet codons (and where U also stands for T) and the amino acid encoded by each codon is described in Lewin (1985). In addition, it is well within the skill of a person trained in the art to create alternative polynucleotide sequences encoding the same, or essentially the same, 2-phenylethanol dehydrogenase enzymes of the subject invention. These variant or alternative polynucleotide sequences are within the scope of the subject invention. As used herein, references to "essentially the same" sequence refers to sequences which encode amino acid substitutions, deletions, additions, or insertions which do not materially alter the functional activity of the polypeptide encoded by the polynucleotides of the present invention. Allelic variants of the nucleotide sequences encoding a 2-phenylethanol dehydrogenase of the invention are also encompassed within the scope of the invention.

The subject invention also concerns an isolated plant 2-phenylethanol dehydrogenase. In one embodiment, the 2-phenylethanol dehydrogenase is a 2-phenylethanol dehydrogenase of

tomato. In a specific embodiment, the 2-phenylethanol dehydrogenase has an amino acid sequence as shown in SEQ ID NO. 2, or an enzymatically functional fragment or variant thereof. A 2-phenylethanol dehydrogenase enzyme of the invention can be purified using standard techniques known in the art. In one embodiment, a polynucleotide of the invention encoding a 2-phenylethanol dehydrogenase is incorporated into a microorganism such as *E. coli* and the 2-phenylethanol dehydrogenase expressed and then isolated therefrom.

Polypeptide fragments according to the subject invention typically comprise a contiguous span of about or at least 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, 105, 106, 107, 108, 109, 110, 111, 112, 113, 114, 115, 116, 117, 118, 119, 120, 121, 122, 123, 124, 125, 126, 127, 128, 129, 130, 131, 132, 133, 134, 135, 136, 137, 138, 139, 140, 141, 142, 143, 144, 145, 146, 147, 148, 149, 150, 151, 152, 153, 154, 155, 156, 157, 158, 159, 160, 161, 162, 163, 164, 165, 166, 167, 168, 169, 170, 171, 172, 173, 174, 175, 176, 177, 178, 179, 180, 181, 182, 183, 184, 185, 186, 187, 188, 189, 190, 191, 192, 193, 194, 195, 196, 197, 198, 199, 200, 201, 202, 203, 204, 205, 206, 207, 208, 209, 210, 211, 212, 213, 214, 215, 216, 217, 218, 219, 220, 221, 222, 223, 224, 225, 226, 227, 228, 229, 230, 231, 232, 233, 234, 235, 236, 237, 238, 239, 240, 241, 242, 243, 244, 245, 246, 247, 248, 249, 250, 251, 252, 253, 254, 255, 256, 257, 258, 259, 260, 261, 262, 263, 264, 265, 266, 267, 268, 269, 270, 271, 272, 273, 274, 275, 276, 277, 278, 279, 280, 281, 282, 283, 284, 285, 286, 287, 288, 289, 290, 291, 292, 293, 294, 295, 296, 297, 298, 299, 300, 301, 302, 303, 304, 305, 306, 307, 308, 309, 310, 311, 312, 313, 314, 315, 316, 317, 318, 319, 320, 321, 322, 323, 324, 325, 326, or 327 amino acids of SEQ ID NO. 2.

Polypeptide fragments of the subject invention can be any integer in length from at least about 25 consecutive amino acids to 1 amino acid less than the sequence shown in SEQ ID NO. 2. Thus, for SEQ ID NO. 2, a polypeptide fragment can be any integer of consecutive amino acids from about 25 to 327 amino acids. The term "integer" is used herein in its mathematical sense and thus representative integers include: 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89,

90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, 105, 106, 107, 108, 109, 110, 111, 112, 113, 114, 115, 116, 117, 118, 119, 120, 121, 122, 123, 124, 125, 126, 127, 128, 129, 130, 131, 132, 133, 134, 135, 136, 137, 138, 139, 140, 141, 142, 143, 144, 145, 146, 147, 148, 149, 150, 151, 152, 153, 154, 155, 156, 157, 158, 159, 160, 161, 162, 163, 164, 165, 166, 167, 168, 169, 170, 171, 172, 173, 174, 175, 176, 177, 178, 179, 180, 181, 182, 183, 184, 185, 186, 187, 188, 189, 190, 191, 192, 193, 194, 195, 196, 197, 198, 199, 200, 201, 202, 203, 204, 205, 206, 207, 208, 209, 210, 211, 212, 213, 214, 215, 216, 217, 218, 219, 220, 221, 222, 223, 224, 225, 226, 227, 228, 229, 230, 231, 232, 233, 234, 235, 236, 237, 238, 239, 240, 241, 242, 243, 244, 245, 246, 247, 248, 249, 250, 251, 252, 253, 254, 255, 256, 257, 258, 259, 260, 261, 262, 263, 264, 265, 266, 267, 268, 269, 270, 271, 272, 273, 274, 275, 276, 277, 278, 279, 280, 281, 282, 283, 284, 285, 286, 287, 288, 289, 290, 291, 292, 293, 294, 295, 296, 297, 298, 299, 300, 301, 302, 303, 304, 305, 306, 307, 308, 309, 310, 311, 312, 313, 314, 315, 316, 317, 318, 319, 320, 321, 322, 323, 324, 325, 326, and/or 327.

Each polypeptide fragment of the subject invention can also be described in terms of its N-terminal and C-terminal positions. For example, combinations of N-terminal to C-terminal fragments of about 25 contiguous amino acids to 1 amino acid less than the full length polypeptide of SEQ ID NO. 2 are included in the present invention. Thus, a 25 consecutive amino acid fragment could correspond to amino acids of SEQ ID NO. 2 selected from the group consisting of 1-25, 2-26, 3-27, 4-28, 5-29, 6-30, 7-31, 8-32, 9-33, 10-34, 11-35, 12-36, 13-37, 14-38, 15-39, 16-40, 17-41, 18-42, 19-43, 20-44, 21-45, 22-46, 23-47, 24-48, 25-49, 26-50, 27-51, 28-52, 29-53, 30-54, 31-55, 32-56, 33-57, 34-58, 35-59, 36-60, 37-61, 38-62, 39-63, 40-64, 41-65, 42-66, 43-67, 44-68, 45-69, 46-70, 47-71, 48-72, 49-73, 50-74, 51-75, 52-76, 53-77, 54-78, 55-79, 56-80, 57-81, 58-82, 59-83, 60-84, 61-85, 62-86, 63-87, 64-88, 65-89, 66-90, 67-91, 68-92, 69-93, 70-94, 71-95, 72-96, 73-97, 74-98, 75-99, 76-100, 77-101, 78-102, 79-103, 80-104, 81-105, 82-106, 83-107, 84-108, 85-109, 86- 110, 87-111, 88,-112, 89-113, 90-114, 91-115, 92-116, 93-117, 94-118, 95-119, 96-120, 97-121, 98-122, 99-123, 100-124, 101-125, 102-126, 103-127, 104-128, 105-129, 106-130, 107-131, 108-132, 109-133, 110-134, 111-135, 112-136, 113-137, 114-138, 115-139, 116-140, 117-141, 118-142, 119-143, 120-144, 121-145, 122-146, 123-147, 124-148, 125-149, 126-150, 127-151, 128-152, 129-153, 130-154, 131-155, 132-156, 133-157, 134-158, 135-159, 136-160, 137-161, 138-162, 139-163, 140-164, 141-165, 142-166,

143-167, 144-168, 145-169, 146-170, 147-171, 148-172, 149-173, 150-174, 151-175, 152-176, 153-177, 154-178, 155-179, 156-180, 157-181, 158-182, 159-183, 160-184, 161-185, 162-186, 163-187, 164-188, 165-189, 166-190, 167-191, 168-192, 169-193, 170-194, 171-195, 172-196, 173-197, 174-198, 175-199, 176-200, 177-201, 178-202, 179-203, 180-204, 181-205, 182-206, 183-207, 184-208, 185-209, 186-210, 187-211, 188-212, 189-213, 190-214, 191-215, 192-216, 193-217, 194-218, 195-219, 196-220, 197-221, 198-222, 199-223, 200-224, 201-225, 202-226, 203-227, 204-228, 205-229, 206-230, 207-231, 208-232, 209-233, 210-234, 211-235, 212-236, 213-237, 214-238, 215-239, 216-240, 217-241, 218-242, 219-243, 220-244, 221-245, 222-246, 223-247, 224-248, 225-249, 226-250, 227-251, 228-252, 229-253, 230-254, 231-255, 232-256, 233-257, 234-258, 235-259, 236-260, 237-261, 238-262, 239-263, 240-264, 241-265, 242-266, 243-267, 244-268, 245-269, 246-270, 247-271, 248-272, 249-273, 250-274, 251-275, 252-276, 253-277, 254-278, 255-279, 256-280, 257-281, 258-282, 259-283, 260-284, 261-285, 262-286, 263-287, 264-288, 265-289, 266-290, 267-291, 268-292, 269-293, 270-294, 271-295, 272-296, 273-297, 274-298, 275-299, 276-300, 277-301, 278-302, 279-303, 280-304, 281-305, 282-306, 283-307, 284-308, 285-309, 286-310, 287-311, 288-312, 289-313, 290-314, 291-315, 292-316, 293-317, 294-318, 295-319, 296-320, 297-321, 298-322, 299-323, 300-324, 301-325, 302-326, 303-327, and 304-328. Similarly, the amino acids corresponding to all other fragments of sizes between 26 consecutive amino acids and 327 consecutive amino acids of SEQ ID NO. 2 are included in the present invention and can also be immediately envisaged based on these examples. Therefore, additional examples, illustrating various fragments of the polypeptides of SEQ ID NO. 2 are not individually listed herein in order to avoid unnecessarily lengthening the specification.

Polypeptide fragments comprising: 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, 105, 106, 107, 108, 109, 110, 111, 112, 113, 114, 115, 116, 117, 118, 119, 120, 121, 122, 123, 124, 125, 126, 127, 128, 129, 130, 131, 132, 133, 134, 135, 136, 137, 138, 139, 140, 141, 142, 143, 144, 145, 146, 147, 148, 149, 150, 151, 152, 153, 154, 155, 156, 157, 158, 159, 160, 161, 162, 163, 164, 165, 166, 167, 168, 169, 170, 171, 172, 173, 174, 175, 176, 177, 178, 179, 180, 181, 182, 183, 184, 185, 186, 187,

188, 189, 190, 191, 192, 193, 194, 195, 196, 197, 198, 199, 200, 201, 202, 203, 204, 205, 206, 207, 208, 209, 210, 211, 212, 213, 214, 215, 216; 217, 218, 219, 220, 221, 222, 223, 224, 225, 226, 227, 228, 229, 230, 231, 232, 233, 234, 235, 236, 237, 238, 239, 240, 241, 242, 243, 244, 245, 246, 247, 248, 249, 250, 251, 252, 253, 254, 255, 256, 257, 258, 259, 260, 261, 262, 263, 264, 265, 266, 267, 268, 269, 270, 271, 272, 273, 274, 275, 276, 277, 278, 279, 280, 281, 282, 283, 284, 285, 286, 287, 288, 289, 290, 291, 292, 293, 294, 295, 296, 297, 298, 299, 300, 301, 302, 303, 304, 305, 306, 307, 308, 309, 310, 311, 312, 313, 314, 315, 316, 317, 318, 319, 320, 321, 322, 323, 324, 325, 326, or 327 consecutive amino acids of SEQ ID NO. 2 may alternatively be described by the formula "n to c" (inclusive), where "n" equals the N-terminal amino acid position and "c" equals the C-terminal amino acid position of the polypeptide. In this embodiment of the invention, "n" is an integer having a lower limit of 1 and an upper limit of the total number of amino acids of the full length polypeptide minus 24 (*e.g.*, 328-24=304 for SEQ ID NO. 2). "c" is an integer between 25 and the number of amino acids of the full length polypeptide sequence (328 for SEQ ID NO. 2) and "n" is an integer smaller then "c" by at least 24. Therefore, for SEQ ID NO. 2, "n" is any integer selected from the list consisting of: 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, 105, 106, 107, 108, 109, 110, 111, 112, 113, 114, 115, 116, 117, 118, 119, 120, 121, 122, 123, 124, 125, 126, 127, 128, 129, 130, 131, 132, 133, 134, 135, 136, 137, 138, 139, 140, 141, 142, 143, 144, 145, 146, 147, 148, 149, 150, 151, 152, 153, 154, 155, 156, 157, 158, 159, 160, 161, 162, 163, 164, 165, 166, 167, 168, 169, 170, 171, 172, 173, 174, 175, 176, 177, 178, 179, 180, 181, 182, 183, 184, 185, 186, 187, 188, 189, 190, 191, 192, 193, 194, 195, 196, 197, 198, 199, 200, 201, 202, 203, 204, 205, 206, 207, 208, 209, 210, 211, 212, 213, 214, 215, 216, 217, 218, 219, 220, 221, 222, 223, 224, 225, 226, 227, 228, 229, 230, 231, 232, 233, 234, 235, 236, 237, 238, 239, 240, 241, 242, 243, 244, 245, 246, 247, 248, 249, 250, 251, 252, 253, 254, 255, 256, 257, 258, 259, 260, 261, 262, 263, 264, 265, 266, 267, 268, 269, 270, 271, 272, 273, 274, 275, 276, 277, 278, 279, 280, 281, 282, 283, 284, 285, 286, 287, 288, 289, 290, 291, 292, 293, 294, 295, 296, 297, 298, 299, 300, 301, 302, 303, and 304 and "c" is any integer selected from the group

consisting of: 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, 105, 106, 107, 108, 109, 110, 111, 112, 113, 114, 115, 116, 117, 118, 119, 120, 121, 122, 123, 124, 125, 126, 127, 128, 129, 130, 131, 132, 133, 134, 135, 136, 137, 138, 139, 140, 141, 142, 143, 144, 145, 146, 147, 148, 149, 150, 151, 152, 153, 154, 155, 156, 157, 158, 159, 160, 161, 162, 163, 164, 165, 166, 167, 168, 169, 170, 171, 172, 173, 174, 175, 176, 177, 178, 179, 180, 181, 182, 183, 184, 185, 186, 187, 188, 189, 190, 191, 192, 193, 194, 195, 196, 197, 198, 199, 200, 201, 202, 203, 204, 205, 206, 207, 208, 209, 210, 211, 212, 213, 214, 215, 216, 217, 218, 219, 220, 221, 222, 223, 224, 225, 226, 227, 228, 229, 230, 231, 232, 233, 234, 235, 236, 237, 238, 239, 240, 241, 242, 243, 244, 245, 246, 247, 248, 249, 250, 251, 252, 253, 254, 255, 256, 257, 258, 259, 260, 261, 262, 263, 264, 265, 266, 267, 268, 269, 270, 271, 272, 273, 274, 275, 276, 277, 278, 279, 280, 281, 282, 283, 284, 285, 286, 287, 288, 289, 290, 291, 292, 293, 294, 295, 296, 297, 298, 299, 300, 301, 302, 303, 304, 305, 306, 307, 308, 309, 310, 311, 312, 313, 314, 315, 316, 317, 318, 319, 320, 321, 322, 323, 324, 325, 326, 327, and 328 provided that "n" is a value less than "c" by at least 24. Every combination of "n" and "c" positions are included as specific embodiments of polypeptide fragments of the invention. All ranges used to describe any polypeptide fragment embodiment of the present invention are inclusive unless specifically set forth otherwise.

Fragments of a plant 2-phenylethanol dehydrogenase of the invention, as described herein, can be obtained by cleaving the polypeptides of the invention with a proteolytic enzyme (such as trypsin, chymotrypsin, or collagenase) or with a chemical reagent, such as cyanogen bromide (CNBr). Alternatively, polypeptide fragments can be generated in a highly acidic environment, for example at pH 2.5. Polypeptide fragments can also be prepared by chemical synthesis or using host cells transformed with an expression vector comprising a polynucleotide encoding a fragment of a 2-phenylethanol dehydrogenase enzyme of the invention, for example, a 2-phenylethanol dehydrogenase that is a fragment of the amino acid sequence shown in SEQ ID NO. 2.

Substitution of amino acids other than those specifically exemplified or naturally present in a plant 2-phenylethanol dehydrogenase enzyme of the invention are also contemplated within

the scope of the present invention. For example, non-natural amino acids can be substituted for the amino acids of 2-phenylethanol dehydrogenase, so long as the 2-phenylethanol dehydrogenase enzyme having the substituted amino acids retains substantially the same biological activity as the 2-phenylethanol dehydrogenase in which amino acids have not been substituted. Examples of non-natural amino acids include, but are not limited to, ornithine, citrulline, hydroxyproline, homoserine, phenylglycine, taurine, iodotyrosine, 2,4-diaminobutyric acid, α-amino isobutyric acid, 4-aminobutyric acid, 2-amino butyric acid, γ-amino butyric acid, ε-amino hexanoic acid, 6-amino hexanoic acid, 2-amino isobutyric acid, 3-amino propionic acid, norleucine, norvaline, sarcosine, homocitrulline, cysteic acid, τ-butylglycine, τ-butylalanine, phenylglycine, cyclohexylalanine, β-alanine, fluoro-amino acids, designer amino acids such as β-methyl amino acids, C-methyl amino acids, N-methyl amino acids, and amino acid analogues in general. Non-natural amino acids also include amino acids having derivatized side groups. Furthermore, any of the amino acids in the protein can be of the D (dextrorotary) form or L (levorotary) form. Allelic variants of a protein sequence of 2-phenylethanol dehydrogenase enzyme used in the present invention are also encompassed within the scope of the invention.

Amino acids can be generally categorized in the following classes: non-polar, uncharged polar, basic, and acidic. Conservative substitutions whereby an 2-phenylethanol dehydrogenase enzyme of the present invention having an amino acid of one class is replaced with another amino acid of the same class fall within the scope of the subject invention so long as the 2-phenylethanol dehydrogenase enzyme having the substitution still retains substantially the same biological activity as the 2-phenylethanol dehydrogenase enzyme that does not have the substitution. Polynucleotides encoding a 2-phenylethanol dehydrogenase enzyme having one or more amino acid substitutions in the sequence are contemplated within the scope of the present invention. Table 3 below provides a listing of examples of amino acids belonging to each class.

Table 3.

Class of Amino Acid	Examples of Amino Acids
Nonpolar	Ala, Val, Leu, Ile, Pro, Met, Phe, Trp
Uncharged Polar	Gly, Ser, Thr, Cys, Tyr, Asn, Gln
Acidic	Asp, Glu
Basic	Lys, Arg, His

The subject invention also concerns variants of the polynucleotides of the present invention that encode enzymatically active 2-phenylethanol dehydrogenase enzymes of the invention. Variant sequences include those sequences wherein one or more nucleotides of the sequence have been substituted, deleted, and/or inserted. The nucleotides that can be substituted for natural nucleotides of DNA have a base moiety that can include, but is not limited to, inosine, 5-fluorouracil, 5-bromouracil, hypoxanthine, 1-methylguanine, 5-methylcytosine, and tritylated bases. The sugar moiety of the nucleotide in a sequence can also be modified and includes, but is not limited to, arabinose, xylulose, and hexose. In addition, the adenine, cytosine, guanine, thymine, and uracil bases of the nucleotides can be modified with acetyl, methyl, and/or thio groups. Sequences containing nucleotide substitutions, deletions, and/or insertions can be prepared and tested using standard techniques known in the art.

Fragments and variants of the 2-phenylethanol dehydrogenase of the present invention can be generated as described herein and tested for the presence of enzymatic function using standard techniques known in the art. For example, the conversion of phenylacetaldehyde to 2-phenylethanol can be assayed according to the present invention. Thus, an ordinarily skilled artisan can readily prepare and test fragments and variants of a 2-phenylethanol dehydrogenase of the invention and determine whether the fragment or variant retains functional enzymatic activity relative to full-length or a wildtype plant 2-phenylethanol dehydrogenase.

Polynucleotides and polypeptides contemplated within the scope of the subject invention can also be defined in terms of more particular identity and/or similarity ranges with those sequences of the invention specifically exemplified herein. The sequence identity will typically

be greater than 60%, preferably greater than 75%, more preferably greater than 80%, even more preferably greater than 90%, and can be greater than 95%. The identity and/or similarity of a sequence can be 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, or 99% as compared to a sequence exemplified herein. Unless otherwise specified, as used herein percent sequence identity and/or similarity of two sequences can be determined using the algorithm of Karlin and Altschul (1990), modified as in Karlin and Altschul (1993). Such an algorithm is incorporated into the NBLAST and XBLAST programs of Altschul *et al.* (1990). BLAST searches can be performed with the NBLAST program, score = 100, wordlength = 12, to obtain sequences with the desired percent sequence identity. To obtain gapped alignments for comparison purposes, Gapped BLAST can be used as described in Altschul *et al.* (1997). When utilizing BLAST and Gapped BLAST programs, the default parameters of the respective programs (NBLAST and XBLAST) can be used. *See* NCBI/NIH website.

The subject invention also contemplates those polynucleotide molecules having sequences which are sufficiently homologous with the polynucleotide sequences exemplified herein so as to permit hybridization with that sequence under standard stringent conditions and standard methods (Maniatis *et al.*, 1982). As used herein, "stringent" conditions for hybridization refers to conditions wherein hybridization is typically carried out overnight at 20-25 C below the melting temperature (Tm) of the DNA hybrid in 6x SSPE, 5x Denhardt's solution, 0.1% SDS, 0.1 mg/ml denatured DNA. The melting temperature, Tm, is described by the following formula (Beltz *et al.*, 1983):

Tm=81.5 C+16.6 Log[Na+]+0.41(%G+C)-0.61(% formamide)-600/length of duplex in base pairs.

Washes are typically carried out as follows:

(1) Twice at room temperature for 15 minutes in 1x SSPE, 0.1% SDS (low stringency wash).

(2) Once at Tm-20 C for 15 minutes in 0.2x SSPE, 0.1% SDS (moderate stringency wash).

As used herein, the terms "nucleic acid" and "polynucleotide" refer to a deoxyribonucleotide, ribonucleotide, or a mixed deoxyribonucleotide and ribonucleotide

polymer in either single- or double-stranded form, and unless otherwise limited, would encompass known analogs of natural nucleotides that can function in a similar manner as naturally-occurring nucleotides. The polynucleotide sequences include the DNA strand sequence that is transcribed into RNA and the strand sequence that is complementary to the DNA strand that is transcribed. The polynucleotide sequences also include both full-length sequences as well as shorter sequences derived from the full-length sequences. Allelic variations of the exemplified sequences also fall within the scope of the subject invention. The polynucleotide sequence includes both the sense and antisense strands either as individual strands or in the duplex.

The subject invention also concerns cells transformed with a polynucleotide of the present invention encoding a 2-phenylethanol dehydrogenase of the invention. In one embodiment, the cell is transformed with a polynucleotide sequence comprising a sequence encoding the amino acid sequence shown in SEQ ID NO. 2, or an enzymatically functional fragment or variant thereof. In a specific embodiment, the cell is transformed with a polynucleotide sequence shown in SEQ ID NO. 1, or a sequence encoding an enzymatically functional fragment or variant of SEQ ID NO. 2. In one embodiment, the polynucleotide encoding the amino acid sequence shown in SEQ ID NO. 2 comprises nucleotides 172 to 1155 of the nucleotide sequence shown in SEQ ID NO. 1. Preferably, the polynucleotide sequence is provided in an expression construct of the invention. The transformed cell can be a prokaryotic cell, for example, a bacterial cell such as *E. coli* or *B. subtilis*, or the transformed cell can be a eukaryotic cell, for example, a plant cell, including protoplasts, or an animal cell. Plant cells include, but are not limited to, dicotyledonous, monocotyledonous, and conifer cells. In one embodiment, the plant cell is a cell from tomato. Animal cells include human cells, mammalian cells, avian cells, and insect cells. Mammalian cells include, but are not limited to, COS, 3T3, and CHO cells.

Plants, plant tissues, and plant cells transformed with or bred to contain a polynucleotide of the invention are also contemplated by the present invention. Plants within the scope of the present invention include monocotyledonous plants, such as, for example, rice, wheat, barley, oats, sorghum, maize, sugarcane, pineapple, onion, bananas, coconut, lilies, grasses, and millet. Plants within the scope of the present invention also include dicotyledonous plants, such as, for

example, tomato, peas, alfalfa, melon, chickpea, chicory, clover, kale, lentil, soybean, tobacco, potato, sweet potato, radish, cabbage, rape, apple trees, grape, cotton, sunflower, and lettuce; and conifers. Preferably, the plant, plant tissue, or plant cell is tomato. Ornamental and herb plants containing a polynucleotide of the invention are also contemplated within the scope of the invention. Ornamental plants include roses, petunia, carnations, orchids, tulips, and the like. Herb plants include parsley, sage, rosemary, thyme, and the like. Techniques for transforming plant cells with a gene are known in the art and include, for example, *Agrobacterium* infection, biolistic methods, electroporation, calcium chloride treatment, *etc*. Transformed cells can be selected, redifferentiated, and grown into plants using standard methods known in the art. The seeds and progeny of any transformed or transgenic plant cells or plants of the invention are also included within the scope of the present invention.

The subject invention also concerns methods for providing a plant with increased flavor or fragrance by incorporating a polynucleotide of the present invention in the genome of the plant cells and expressing the polypeptide encoded by the polynucleotide. In one embodiment, a plant is grown from a transformed plant cell of the invention. Preferably, the polynucleotide encodes a 2-phenylethanol dehydrogenase derived from the same plant species as the plant. In one embodiment, the plant is tomato. In another embodiment, the plant is a rose or other scented ornamental. In a specific embodiment, a polynucleotide encoding an amino acid sequence shown in SEQ ID NO. 2, or an enzymatically functional fragment or variant thereof, is incorporated into a tomato plant genome. In a specific embodiment, the polynucleotide comprises a nucleotide sequence shown in SEQ ID NO. 1, or a sequence encoding an enzymatically functional fragment or variant of SEQ ID NO. 2. In one embodiment, the polynucleotide encoding the amino acid sequence shown in SEQ ID NO. 2 comprises nucleotides 172 to 1155 of the nucleotide sequence shown in SEQ ID NO. 1. The level of expression of a polynucleotide of the invention can be manipulated using standard methods known in the art, including the use of promoters that provide for low, intermediate or high levels of expression.

The subject invention also concerns methods for producing 2-phenylethanol. In one embodiment, recombinantly produced 2-phenylethanol dehydrogenase of the invention can be used to enzymatically convert a suitable substrate, such as phenylacetaldehyde, into 2-

phenylethanol. In another embodiment, a microorganism, such as yeast or *E. coli*, can be transformed with and express a polynucleotide encoding a plant 2-phenylethanol dehydrogenase of the invention and, optionally, one or more enzymes, such as phenylalanine decarboxylase and phenylethylamine oxidase, that through their enzymatic reactions result in a suitable substrate (*e.g.*, phenylacetaldehyde) for 2-phenylethanol dehydrogenase to convert to 2-phenylethanol. Transformed microorganisms can be grown and polynucleotides expressed constitutively or induced, and 2-phenylethanol isolated from the microorganisms.

All patents, patent applications, provisional applications, and publications referred to or cited herein are incorporated by reference in their entirety, including all figures and tables, to the extent they are not inconsistent with the explicit teachings of this specification.

Materials and Methods

Plant material.

Tomato (*Lycopersicon esculentum* Mill. cv. M82) and *Lycopersicon pennellii* introgression lines 8-2 and 8-2-1 (Eshed and Zamir, 1994) were grown in the greenhouse or field under standard conditions. Petunia plants were grown in a greenhouse under standard conditions.

Volatile collection.

Volatiles were collected from approximately 100g of chopped ripe tomato fruit as described by Schmelz *et al.* (2003). Petunia volatiles were collected from five flowers from each plant harvested at dusk. Volatiles were separated on an Agilent DB-5 column and analyzed on an Agilent 6890N gas chromatograph.

Microarrays.

Tomato cDNA microarrays were as described in Moore *et al.* (2002). Total RNAs were isolated as described earlier (Ciardi *et al.*, 2000). Arrays were hybridized with Cy3 or Cy5 labeled cDNAs from M82 and introgression line 8-2-1 fruit. Arrays were performed multiple times and with dyes reversed to ensure accuracy of the expression data.

2-phenylethanol dehydrogenase expression in *E. coli*.

A full-length 2-phenylethanol dehydrogenase cDNA was cloned by 5' RACE from tomato fruit cDNA using primer 5'-TCCTTGGCCCCACCAAGAGAAAGCAAGTGCTGCGT-3' (SEQ ID NO. 3). Following sequence analysis the full-length cDNA was obtained by PCR. The coding region was cloned into vector pDEST15 containing a GST tag (Invitrogen) by recombination, and transformed into *E. coli* strain BL21-SI (Invitrogen) for inducible protein expression. Enzyme activity of crude *E. coli* extracts was determined by the method of Larroy *et al.* (2002) using phenylacetaldehyde, cinnamaldehyde or vanillin as a substrate.

Production of transgenic petunia plants.

The full-length 2-phenylethanol dehydrogenase cDNA (SEQ ID NO. 1) was cloned in a vector under the control of the figwort mosaic virus 35S promoter (Richins *et al.*, 1987) and followed by the Agrobacterium nopaline synthase (*nos*) 3' terminator. The transgene was introduced into *Petunia hybrida* cv. Mitchell Diploid by the method of Wilkinson *et al.* (1997) with kanamycin resistance as a selectable marker.

Following are examples which illustrate procedures for practicing the invention. These examples should not be construed as limiting. All percentages are by weight and all solvent mixture proportions are by volume unless otherwise noted.

Example 1—Volatiles Analysis

Volatiles analysis by GC on a DB5 column indicated high levels of phenylacetaldehyde and 2-phenylethanol in ripe fruit from L. pennellii introgression line (IL) 8-2-1. Levels of other tomato volatiles were similar to control M82 ripe fruit (Figure 1). Levels of 2-phenylethanol were approximately 250X higher in 8-2-1 fruit than in M82 fruit. Phenylacetaldehyde levels in IL8-2-1 fruit were approximately 20X higher than in control fruit (Figure 2). IL8-2-1 fruit had a distinct floral (rose-like) aroma consistent with the floral aromas of phenylacetaldehyde and 2-phenylethanol.

Example 2—Microarray Analysis

Microarrays containing approximately 3,000 tomato cDNAs indicated that an alcohol dehydrogenase gene (cLET2M9) was more highly expressed in IL8-2-1 fruit than in control M82 fruit; whereas, a related tomato alcohol dehydrogenase gene was not upregulated in IL8-2-1 fruit (Table 4).

Table 4. Microarray gene expression data for two alcohol dehydrogenase–like genes

Microarray clone	Ratio
cLET2M9	+2.2
cLEG71F12	-2.35

RNAs extracted from M82 and introgression line 8-2-1 fruit were compared using cDNA microarrays. Positive values indicate higher RNA expression levels in introgression line 8-2-1 fruit; negative values indicate higher expression in M82 fruit.

Since clone cLET2M9 was only a partial cDNA, the full-length cDNA sequence for this clone was obtained by 5' RACE. The full-length cDNA sequence of 2-phenylethanol dehydrogenase was then obtained by PCR, and confirmed by sequence analysis (Figure 3A). The deduced amino acid sequence of the 2-phenylethanol dehydrogenase is shown in Figure 3B (SEQ ID NO. 2). In plants, a substrate such as phenylacetaldehyde can be converted to 2-phenylethanol by 2-phenylethanol dehydrogenase (Figure 4).

Example 3—Enzyme Activity

The 2-phenylethanol dehydrogenase coding region of the full-length cLET2M9 and the related cLEG71F12 were cloned in vector pDEST15 with a GST tag and transformed into *E. coli* BL21-SI cells for inducible expression. The production of recombinant protein in *E. coli* was determined by Western blotting with an anti-GST antibody. Alcohol dehydrogenase activities on phenylacetaldehyde and several related substrates were determined spectrophotometrically by the reduction in levels of NADPH and a decrease in OD_{340} (Figure 5). The highest level of activity was observed with phenylacetaldehyde as a substrate. Lower activity levels were also observed with cinnamaldehyde as a substrate, whereas negligible activity was seen with vanillin

as a substrate. Protein from cLEG71F12 expressed in *E. coli* showed very little activity on the three substrates tested.

Example 4—Expression of 2-phenylethanol Dehydrogenase in Transgenic Petunia

Full-length tomato 2-phenylethanol dehydrogenase cDNA was introduced into petunia (cv. Mitchell Diploid) under control of the constitutively expressed figwort mosaic virus promoter. Several transgenic petunia lines had high levels of expression of the tomato gene in flowers (data not shown). Wild-type petunia flowers emit relatively high levels of phenylacetaldehyde and lower levels of 2-phenylethanol. However, the transgenic petunia flowers expressing the polynucleotide encoding tomato 2-phenylethanol dehydrogenase have higher levels of 2-phenylethanol and lower levels of phenylacetaldehyde than wild-type flowers (Figure 6). Levels of other petunia flower volatiles were similar to wild-type in the transgenic flowers. A range of phenylacetaldehyde and 2-phenylethanol levels were seen in the transgenic lines, however the majority of the lines had higher levels of 2-phenylethanol and lower levels of phenylacetaldehyde than wild-type flowers (Figure 7). Overall, these data indicate that the introduction of the 2-phenylethanol dehydrogenase tomato transgene results in the conversion of phenylacetaldehyde to 2-phenylethanol in petunia flowers.

It should be understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application. In addition, any elements or limitations of any invention or embodiment thereof disclosed herein can be combined with any and/or all other elements or limitations (individually or in any combination) or any other invention or embodiment thereof disclosed herein, and all such combinations are contemplated with the scope of the invention without limitation thereto.

References

U.S. Patent No. 5,106,739

U.S. Patent No. 5,625,136

U.S. Patent No. 5,034,322

U.S. Patent No. 5,589,610

U.S. Patent No. 5,639,948

U.S. Patent No. 6,462,185

Aharoni, A. *et al.* (2000) "Identification of the SAAT gene involved in strawberry flavor biogenesis by use of DNA microarrays" *Plant Cell* 12:647-661.

Altschul, S. F. *et al.* (1990) "Basic Local Alignment Search Tool" *J. Mol. Biol.* 215:402-410.

Altschul, S. F. *et al.* (1997) "Gapped BLAST and PSI-BLAST: A New Generation of Protein Database Search Programs" *Nucl. Acids Res.* 25:3389-3402.

Baldwin, E.A. *et al.* (2000) "Flavor trivia and tomato aroma: Biochemistry and possible mechanisms for control of important aroma components" *Hortscience* 35:1013-1022.

Bartley, G. *et al.* (1994) "Molecular biology of carotenoid biosynthesis in plants" *Annual Review of Plant Physiology & Plant Molecular Biology* Jones, R. L.; Somerville, C. R.: Eds. Annual Review of Plant Physiology and Plant Molecular Biology. 45: 287-301.

Beltz, G. A., Jacobs, K. A., Eickbush, T. H., Cherbas, P. T., Kafatos, F. C. (1983) "Isolation of multigene families and determination of homologies by filter hybridization methods" *Methods of Enzymology*, R. Wu, L. Grossman and K. Moldave [eds.] Academic Press, New York 100:266-285.

Blume, B. and Grierson, D. (1997) "Expression of ACC oxidase promoter-GUS fusions in tomato and *Nicotiana plumbaginifolia* regulated by developmental and environmental stimuli" *Plant J.* 12: 731-746.

Buttery, R. *et al.* (1988) "Quantitative studies on origins of fresh tomato aroma volatiles" *J. Agric. Food Chem.* 36:1247-1250.

Ciardi, J.A. *et al.* (2000) "Response to *Xanthomonas campestris* pv. *vesicatoria* in tomato involves regulation of ethylene receptor gene expression" *Plant Physiol.* 123:81-92.

Clancy, M. and Hannah, L.C. (2002) "Splicing of the maize Sh1 first intron is essential for enhancement of gene expression, and a T-rich motif increases expression without affecting splicing," *Plant Physiol.* 130(2):918-29.

de Boer, H. A., Comstock, L. J., Vasser, M. (1983) "The tac promoter: a functional hybrid derived from the trp and lac promoters" *Proc. Natl. Acad. Sci. USA* 80(1):21-25.

Deikman, J. *et al.* (1992) "Organization of ripening and ethylene regulatory regions in a fruit-specific promoter from tomato (*Lycopersicon esculentum*)" *Plant Physiol.* 100:2013-2017.

Eshed, Y. and Zamir, D. (1994) "A genomic library of *Lycopersicon pennellii* in *L. esculentum*: A tool for fine mapping of genes" *Euphytica* 79:175-179.

Fray, R. and Grierson, D. (1993) "Identification and genetic analysis of normal and mutant phytoene synthase genes of tomato by sequencing, complementation, and co-suppression" *Plant Mol. Biol.* 22:589-602.

Giovannoni, J. (2001) "Molecular Regulation of Fruit Ripening" *Ann. Rev. Plant Physiol. Plant Molec. Biol.* 52:725-749.

Giovannoni, J. "A MADS-box gene necessary for fruit ripening at the tomato *ripening-inhibitor* (*rin*) locus" *Submitted.*

Giovannoni, J. *et al.* (1999) "Analysis of gene expression and mutants influencing ethylene responses and fruit development in tomato" *In* Biology and Biotechnology of the Plant Hormone Ethylene II, (Kanellis, A. ed.) Kluwer Academic Publishers, pp. 119-127.

Giovannoni, J. *et al.* (1989) "Expression of a chimeric polygalacturonase gene in transgenic rin (ripening inhibitor) tomato fruit results in polyuronide degradation but not fruit softening" *Plant Cell* 1:53-63.

Good, X. *et al.* (1994) "Reduced ethylene synthesis by transgenic tomatoes expressing S-adenosylmethionine hydrolase." *Plant Molec. Biol.* 26:781-790.

Gray, J.E. *et al.* (1994) "The use of transgenic and naturally occurring mutants to understand and manipulate tomato fruit ripening" *Plant, Cell and Environment* 17:557-571.

Hamilton, A. *et al.* (1990) "Antisense gene that inhibits synthesis of the hormone ethylene in transgenic plants" *Nature* 346:284-287.

Hobson, G. and Grierson, D. (1993) "Tomato" In: Seymour GB, Taylor JE, Tucker GA (eds) Biochemistry of Fruit Ripening. Chapman and Hall, London pp 405-442.

Karlin S. and Altschul, S. F. (1990) "Methods for Assessing the Statistical Significance of Molecular Sequence Features by Using General Scoring Schemes" *Proc. Natl. Acad. Sci. USA* 87:2264-2268.

Karlin S. and Altschul, S. F. (1993) "Applications and Statistics for Multiple High-Scoring Segments in Molecular Sequences" *Proc. Natl. Acad. Sci. USA* 90:5873-5877.

Klee, H.J. *et al.* (1991) "Control of ethylene synthesis by expression of a bacterial enzyme in transgenic tomato plants" *Plant Cell* 3:1187-1193.

Kramer, M. *et al.* (1990) "Field evaluation of tomatoes with reduced polygalacturonase by antisense RNA" In Horticultural Biotechnology. Bennett, A. and O.Neill, S. (eds.) Alan R. Liss. pp. 347-355.

Lanahan, M.B. *et al.* (1994) "The Never Ripe mutation blocks ethylene perception in tomato" *Plant Cell* 6:521-530.

Larroy, C. *et al.* (2002) "Characterization of the *Saccharomyces cervisiae YMR318C (ADH6)* gene product as a broad specificity NADPH-dependent alcohol dehydrogenase: relevance in aldehyde reduction" *Biochem. J.* 361:163-172.

Lashbrook, C. *et al.* (1998) "Transgenic analysis of tomato endo-beta-1,4-glucanase gene function. Role of CEL1 in floral abscission" *Plant J.* 13:303-310.

Lelievre, J. *et al.* (1998) "Ethylene and fruit ripening" *Plant Physiol.* 101:727-739.

Lewin, B. (1985) Genes II, John Wiley & Sons, Inc., p. 96.

Lincoln, J. *et al.* (1987) "Regulation of gene expression by ethylene during Lycopersicon esculentum (tomato) fruit development" *Proc. Nat. Acad. Sci. USA*. 84:2793-2797.

Maniatis, T., E.F. Fritsch, J. Sambrook (1982) "Nuclease *Bal*31" *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Maunders, M. *et al.* (1987) "Ethylene stimulates the accumulation of ripening-related mRNAs in tomatoes" *Plant Cell Environ.* 10:177-184.

Moore, S. *et al.* (2002) "Use of genomics tools to isolate key ripening genes and analyse fruit maturation in tomato" *J. Exp. Bot.* 53:2023-2030.

Nakatsuka, A. *et al.* (1998) "Differential expression and internal feedback regulation of 1-aminocyclopropane-1-carboxylate synthase, of 1-aminocyclopropane-1-caboxylate oxidase, and ethylene receptor genes in tomato during development and ripening" *Plant Physiol.* 118:1295-1305.

Oeller, P.W. *et al.* (1991) "Reversible inhibition of tomato fruit senescence by antisense 1-aminocyclopropane-1-carboxylate synthase" *Science* 254:427-439.

Payton, S. *et al.* (1996) "Ethylene receptor expression is regulated during fruit ripening, flower senescence and abscission" *Plant Molecular Biology* 31:1227-1231.

Picton, S. *et al.* (1993) "Altered fruit ripening and leaf senescence in tomatoes expressing an antisense ethylene-forming enzyme transgene" *Plant J.* 3:469-481.

Rhodes, M.J.C. (1980) "The maturation and ripening of fruits" In: KV Thimann (ed) Senescence in Plants. CRC Press, Boca Raton, FL, pp. 157-205.

Richins, R.D. *et al.* (1987) Sequence of the figwort mosaic virus DNA (caulimovirus group)" *Nucl. Acids Res.* 15:8451-8466.

Riley, J. and Thompson, J. (1997) "Subcellular generation and distribution of lipid-derived volatiles in the ripe tomato" *J. Plant Physiol.* 150:546-551.

Ronen, G. *et al.* (1999) "Regulation of carotenoid biosynthesis during tomato fruit development: Expression of the gene for lycopene epsilon-cyclase is down-regulated during ripening and is elevated in the mutant Delta" *Plant J.* 17: 341-351.

Schmelz, E.A. *et al.* (2003) "Qualitative relationships between induced jasmonic acid levels and volatile emission in *Zea mays* during *Spodoptera exigua* herbivory" *Planta* 216:665-673.

Schwartz, S. *et al.* (2001) "Characterization of a novel carotenoid cleavage dioxygenase from plants" *J. Biol. Chem.* 276:25208-25211.

Smith, C. *et al.* (1988) "Antisense RNA inhibition of polygalacturonase gene expression in transgenic tomatoes" *Nature* 334:724-726.

Tan, B. *et al.* (1997) *Proc. Nat. Acad. Sci. USA* 94:12235-12240.

Tanksley, S. *et al.* (1992) "High density molecular linkage maps of the tomato and potato genomes" *Genetics* 132:1141-1160.

Theologis, A. (1993) "Use of a tomato mutant constructed with reverse genetics to study fruit ripening, a complex developmental process" *Dev. Genetics* 14:282-259.

Tucker, G.A. and Brady, C.J. (1987) "Silver ions interrupt tomato fruit ripening" *J. Plant Physiol.* 127:165-169.

Vrebalov, J. *et al.* (2002) "A MADS-box gene necessary for fruit ripening at the tomato *ripening-inhibitor* (*rin*) locus" *Science* 296:343-346.

Wilkinson, J.Q. *et al.* (1997) "A dominant mutant receptor from *Arabidopsis* confers ethylene insensitivity in heterologous plants" *Nature Biotechnol.* 15: 444-447.

Wilkinson, J. *et al.* (1995) "An ethylene-inducible component of signal transduction encoded by Never-ripe" *Science* 270:1807-1809.

Xu, D., McElroy, D., Thornburg, R. W., Wu, R. *et al.* (1993) "Systemic induction of a potato pin2 promoter by wounding, methyl jasmonate, and abscisic acid in transgenic rice plants" *Plant Molecular Biology* 22:573-588.

Yang, S.F. (1985) "Biosynthesis and action of ethylene" *HortScience* 20:41-45.

Yang, T. T. *et al.* (1996) "Optimized Codon Usage and Chromophore Mutations Provide Enhanced Sensitivity with the Green Fluorescent Protein" *Nucleic Acid Research* 24(22):4592-4593.

Yen, H. *et al.* (1997) "The tomato high pigment (hp) locus maps to chromosome 2 and influences plastome copy number and fruit quality" *Theoretical and Applied Genetics* 95:1069-1079.

Yen, H. *et al.* (1995) "The tomato Never-ripe locus regulates ethylene-inducible gene expression and is linked to a homologue of the Arabidopsis ETR1 gene" *Plant Physiology* 107:1343-1353.

Abstract of the Disclosure

The subject invention concerns polynucleotides encoding a plant 2-phenylethanol dehydrogenase enzyme. In one embodiment, the polynucleotide encodes a tomato 2-phenylethanol dehydrogenase. The subject invention also concerns 2-phenylethanol dehydrogenase polypeptides encoded by polynucleotides of the present invention. The subject invention also concerns methods for providing a plant with an increased flavor and aroma volatile. Plants can be transformed with a polynucleotide of the present invention. The subject invention also concerns these transformed plant cells, plant tissue, and plants and transgenic progeny thereof.

FIG. 1A

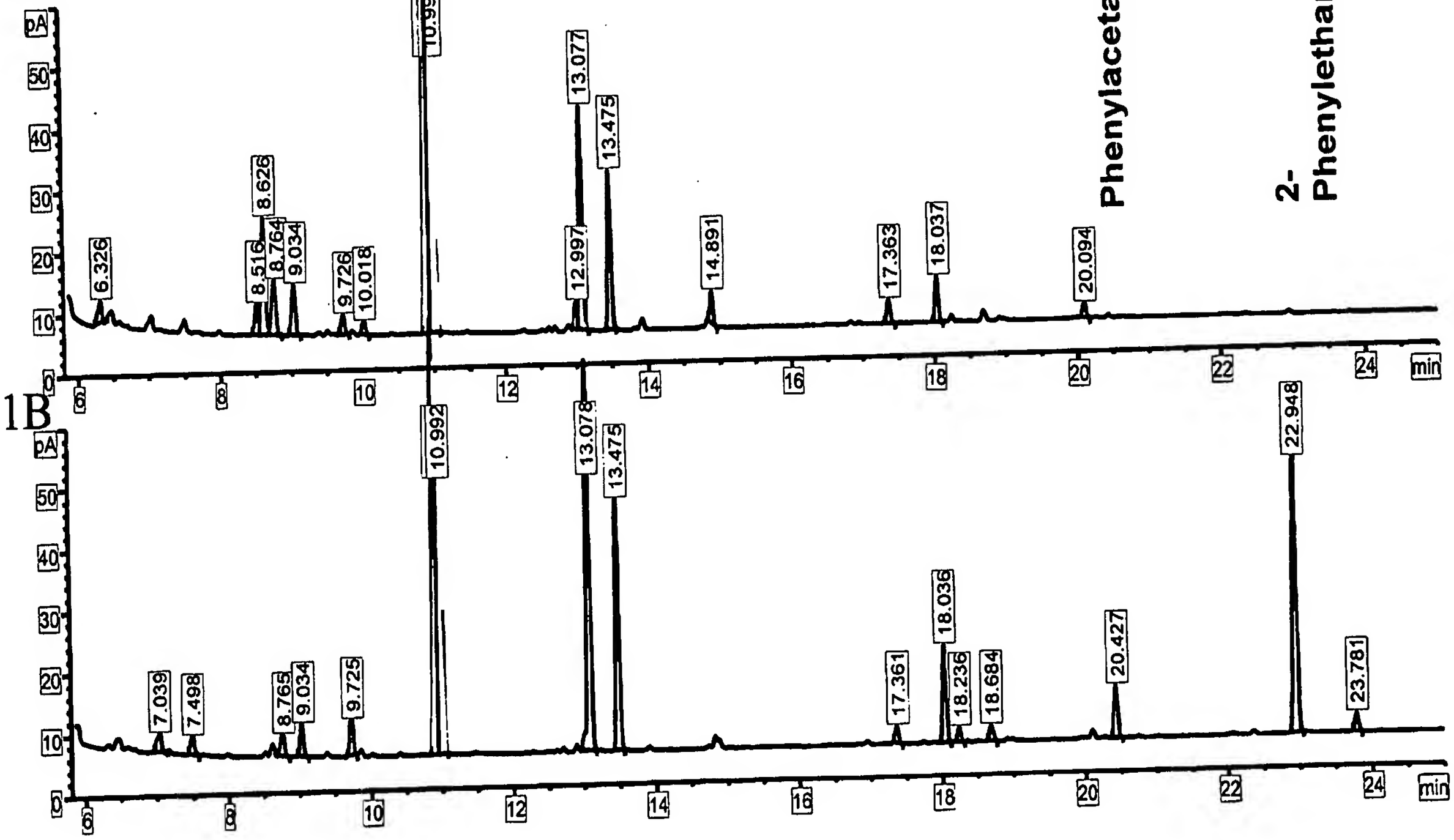


FIG. 1B

Retention time (mins)

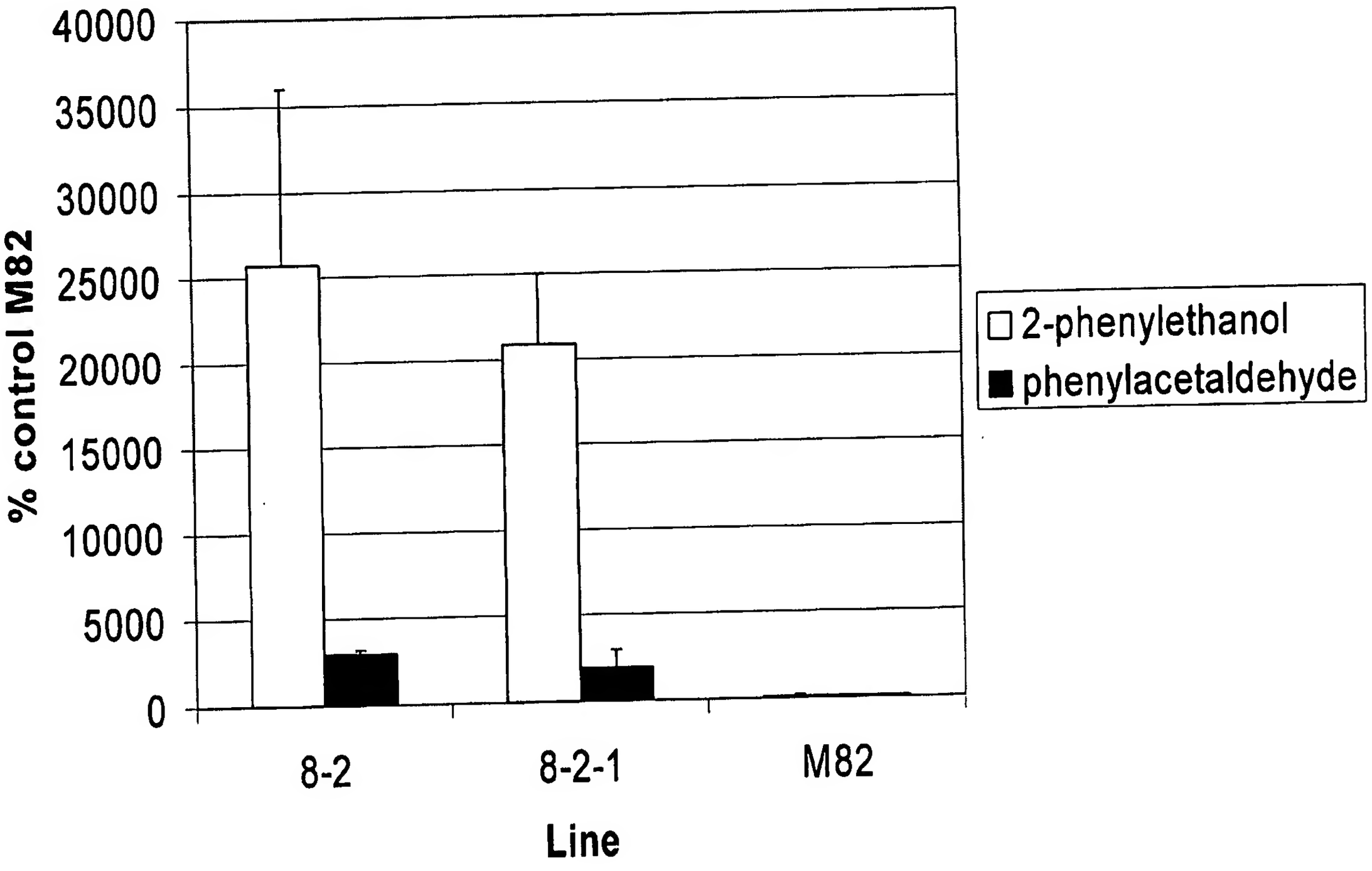

40000
35000
30000
25000
20000
15000
10000
5000
0
% control M82
8-2
8-2-1
M82
Line
2-phenylethanol
phenylacetaldehyde

FIG. 2

```
   1  GCCCTTCTAA TACGACTCAC TATAGGGCAA GCAGTGGTAA CAACGCAGAG
  51  TACGCGGGGG AAGGATAATC TCTCAAATTA CTTTCTTTTT TTTTCCTATC
 101  AATTCTTTAT ACCAAAATAA TATTATTGTT TTTTTCTCCT CTGTTTCTGC
 151  TTCGTATTTT TGCTGAGAGA AATGAGTGTG ACAGCGAAAA CAGTGTGTGT
 201  AACAGGAGCT TCAGGTTACA TAGCTTCATG GCTAGTCAAA TTCTTGCTAC
 251  ATAGTGGTTA CAATGTGAAG GCTTCTGTTC GTGATCCAAA TGATCCCAAG
 301  AAAACGCAGC ACTTGCTTTC TCTTGGTGGG GCCAAGGAGA GGCTTCACTT
 351  GTTCAAAGCA AACCTATTAG AAGAAGGTTC ATTTGATGCT GTAGTTGATG
 401  GATGTGAAGG TGTATTCCAT ACAGCGTCTC CTTTTTACTA CTCTGTTACA
 451  GACCCACAGG CTGAATTACT TGATCCTGCT GTTAAGGGAA CACTCAATCT
 501  TCTCGGGTCA TGTGCCAAAG CACCATCAGT AAAACGAGTT GTTTTAACGT
 551  CTTCCATAGC TGCAGTTGCT TACAGTGGTC AGCCTCGGAC ACCTGAGGTT
 601  GTGGTTGATG AGAGCTGGTG GACCAGTCCA GACTACTGCA AAGAAAAACA
 651  GCTCTGGTAT GTCCTCTCAA AGACATTGGC TGAGGATGCT GCGTGGAAGT
 701  TTGTGAAGGA GAAAGGCATT GATATGGTTG TAGTAAACCC TGCTATGGTT
 751  ATTGGTCCTC TGTTACAGCC TACACTTAAT ACCAGTTCTG CTGCAGTCTT
 801  GAGCTTGGTA AATGGTGCTG AGACATACCC AAATTCCTCT TTTGGGTGGG
 851  TTAACGTGAA AGATGTTGCA AATGCACATA TTCTTGCATT TGAGAACCCT
 901  TCAGCTAATG GGAGATACTT AATGGTTGAG AGGGTTGCAC ACTATTCTGA
 951  TATATTGAAG ATATTGCGTG ACCTTTATCC TACTATGCAA CTTCCAGAAA
1001 AGTGTGCTGA TGACAACCCA TTGATGCAAA ATTATCAAGT ATCAAAGGAG
1051 AAGGCAAAAA GCTTGGGTAT TGAGTTTACT ACCCTTGAAG AAAGCATCAA
1101 AGAAACTGTT GAAAGTTTGA AGGAAAAGAA GTTTTTTGGA GGTTCATCTT
1151 CTATGTAAAA GGCTTCTCAA AGCTTTTATG GTTTTGTTGA ACAATACTAC
1201 CCACCCCACC CTACCCTACA CACTTTTTTT TTTTACTTCT TTTAGCTAAT
1251 TATAGAATCA AGAAGTCGAA TGGTATATCC GTTAATAAAT TTCGATCAGA
1301 TGAGGTTGAA ATTTGTTCTA TATCTAGAGA TTTTTACAGA CTGGTTTGAT
                1351  AGAAAAAAAA AAAAAAA
```

FIG. 3A

```
  1  MSVTAKTVCV TGASGYIASW LVKFLLHSGY NVKASVRDPN DPKKTQHLLS
 51  LGGAKERLHL FKANLLEEGS FDAVVDGCEG VFHTASPFYY SVTDPQAELL
101   DPAVKGTLNL LGSCAKAPSV KRVVLTSSIA AVAYSGQPRT PEVVVDESWW
151   TSPDYCKEKQ LWYVLSKTLA EDAAWKFVKE KGIDMVVVNP AMVIGPLLQP
201   TLNTSSAAVL SLVNGAETYP NSSFGWVNVK DVANAHILAF ENPSANGRYL
251   MVERVAHYSD ILKILRDLYP TMQLPEKCAD DNPLMQNYQV SKEKAKSLGI
301   EFTTLEESIK ETVESLKEKK FFGGSSSM
```

FIG. 3B

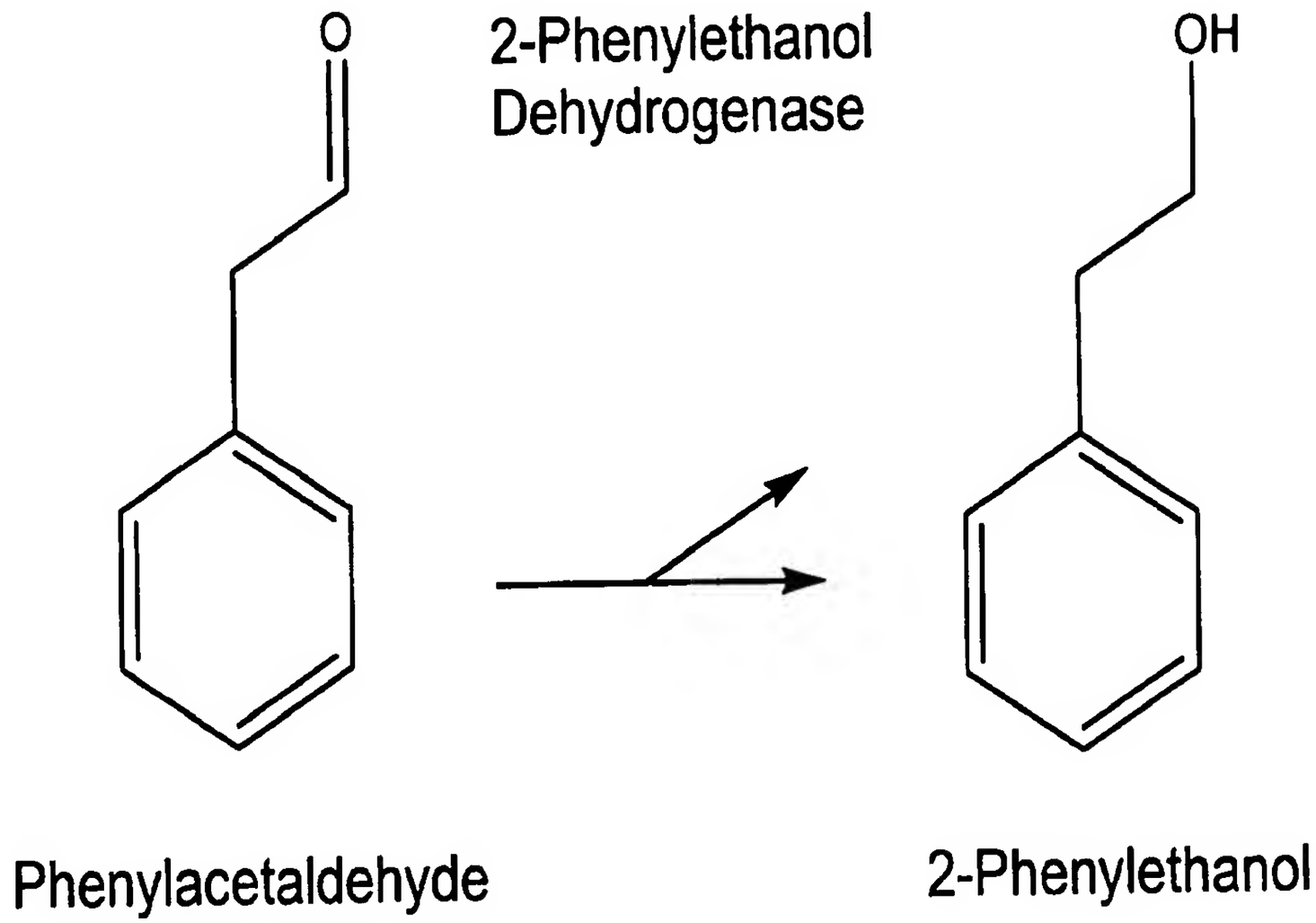

O
2-Phenylethanol
Dehydrogenase
OH
Phenylacetaldehyde
2-Phenylethanol

FIG. 4

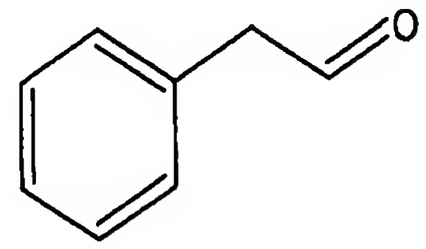


FIG. 5A-1

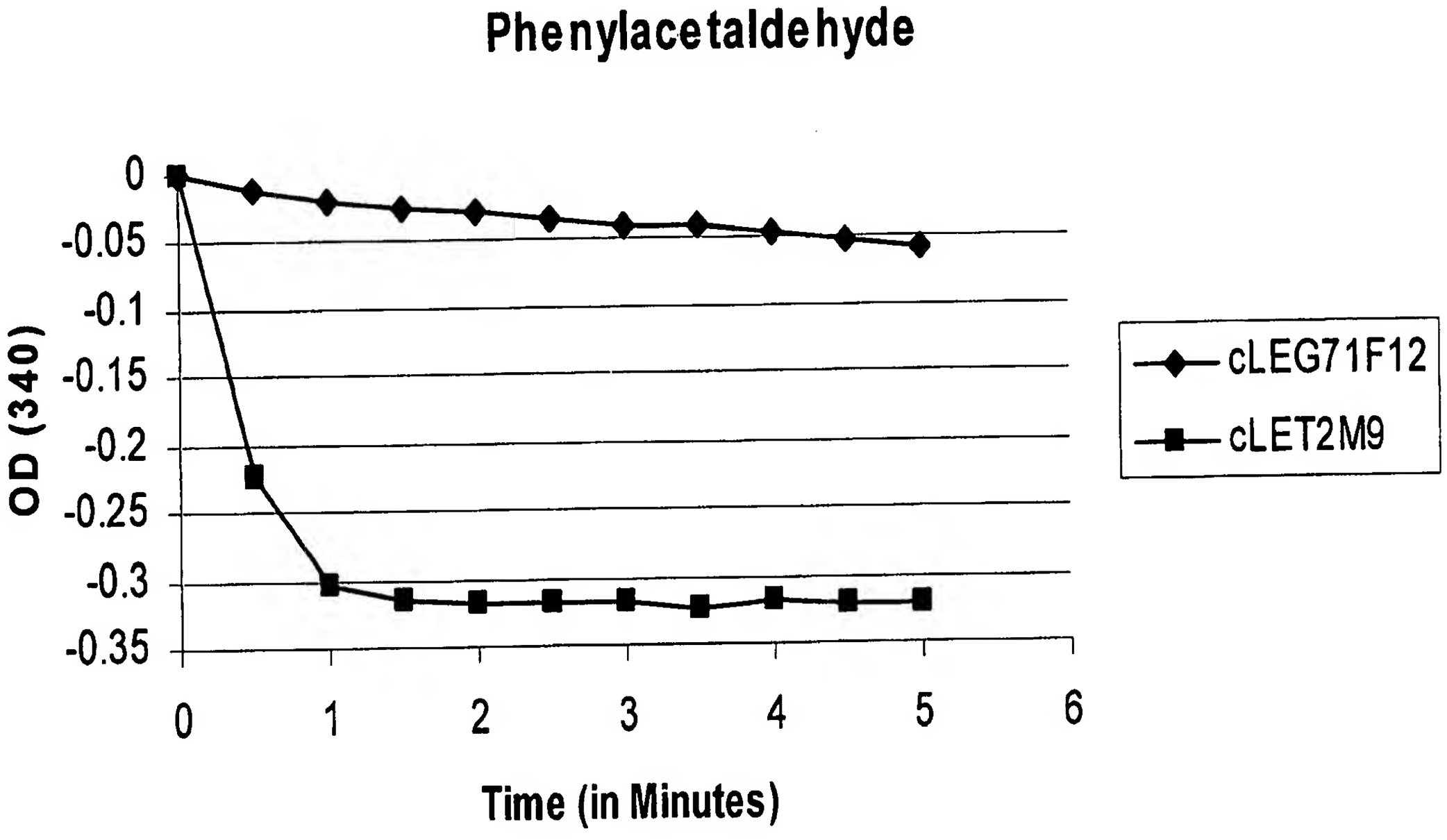


FIG. 5A

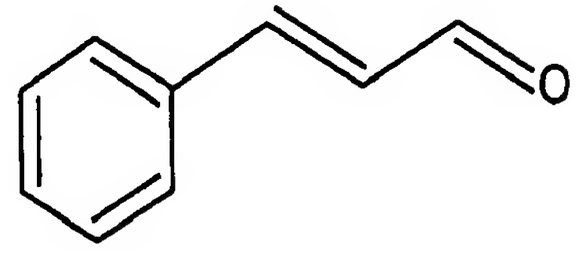


FIG. 5B-1

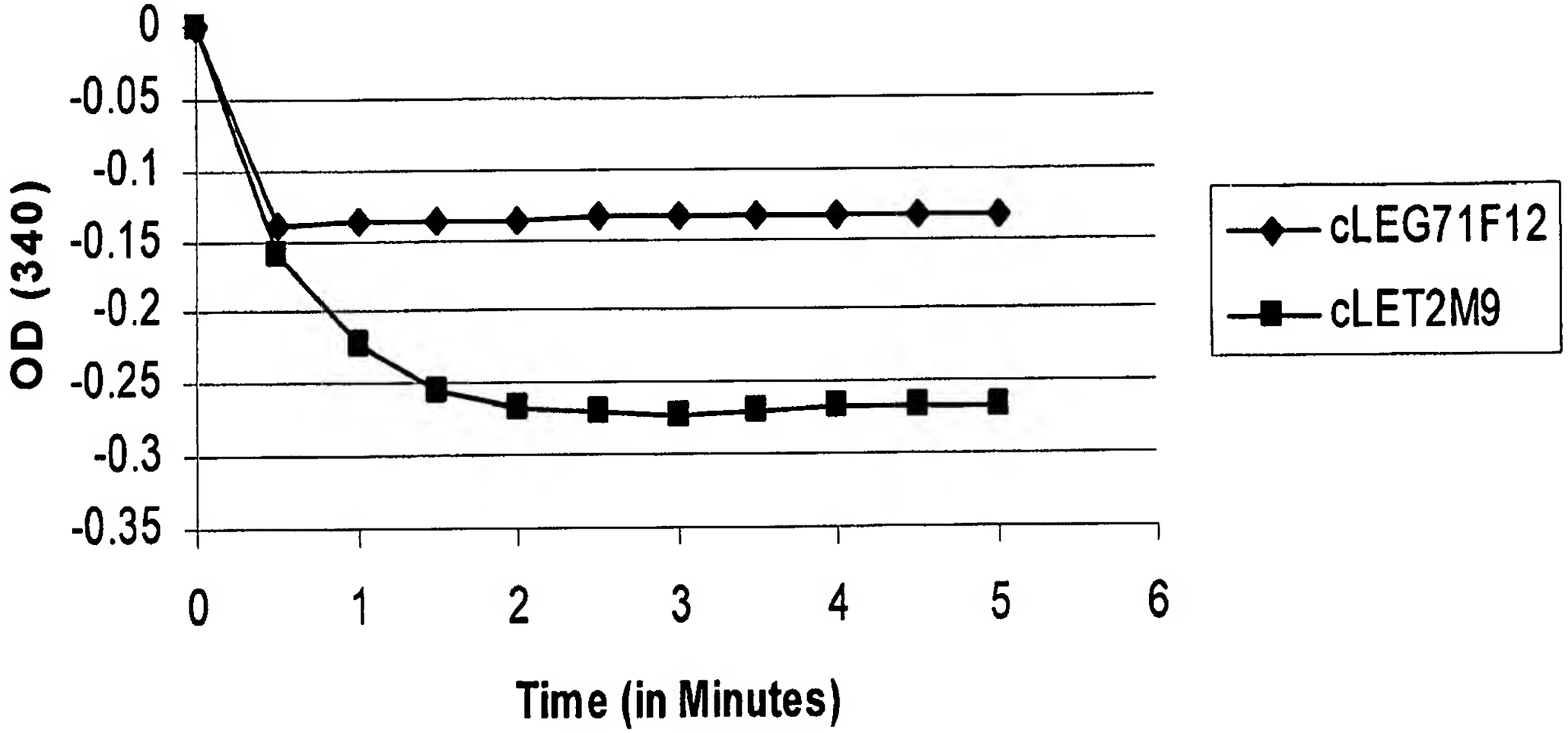


FIG. 5B

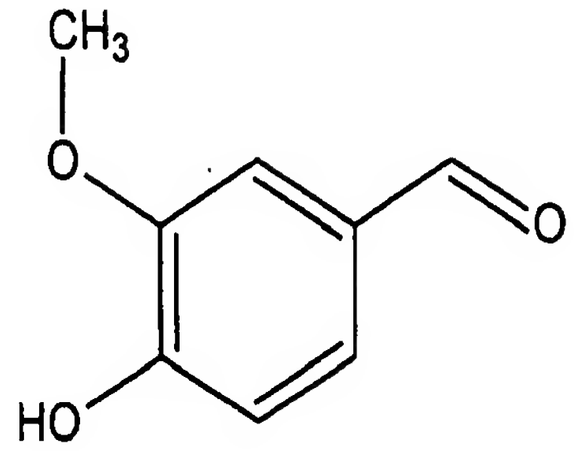


FIG. 5C-1

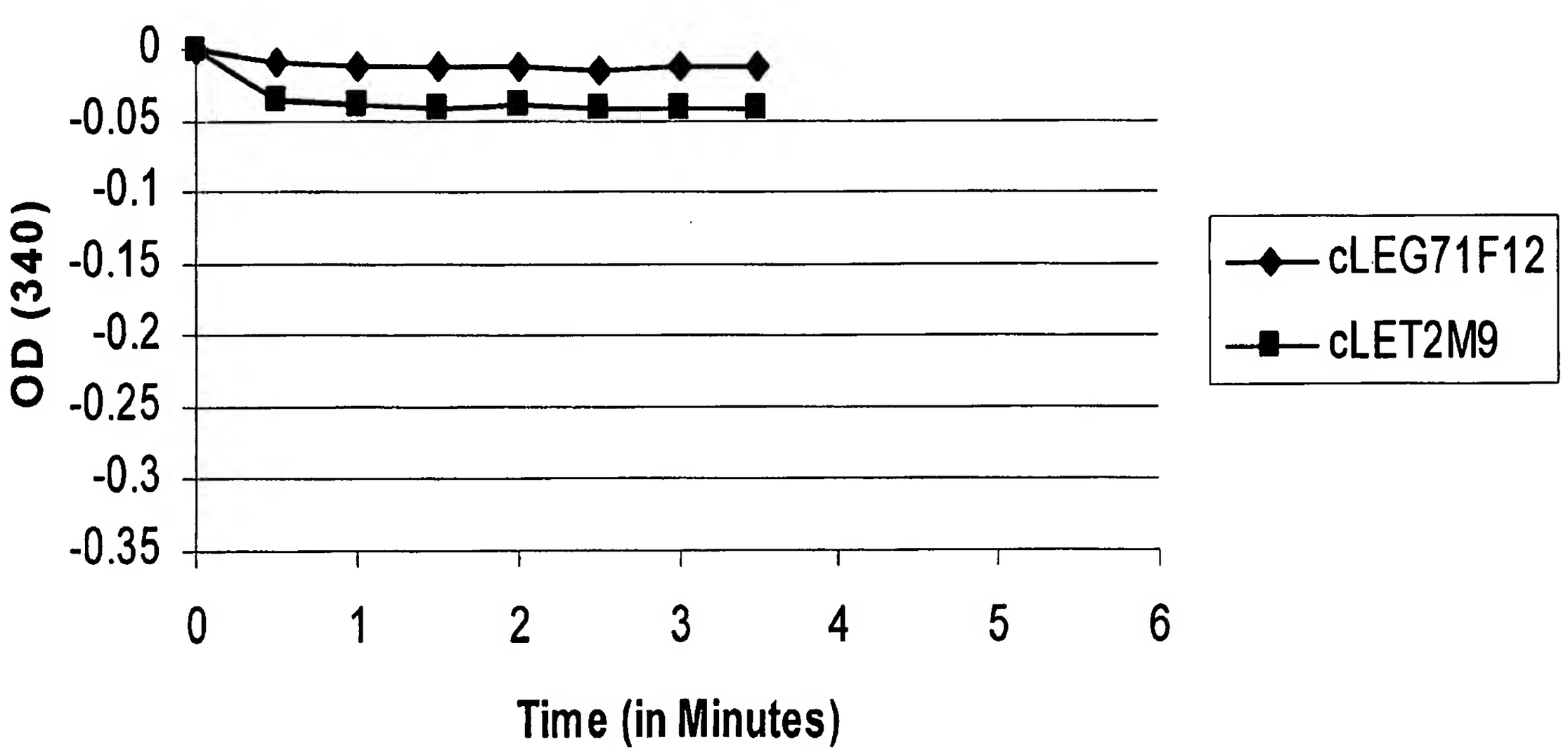
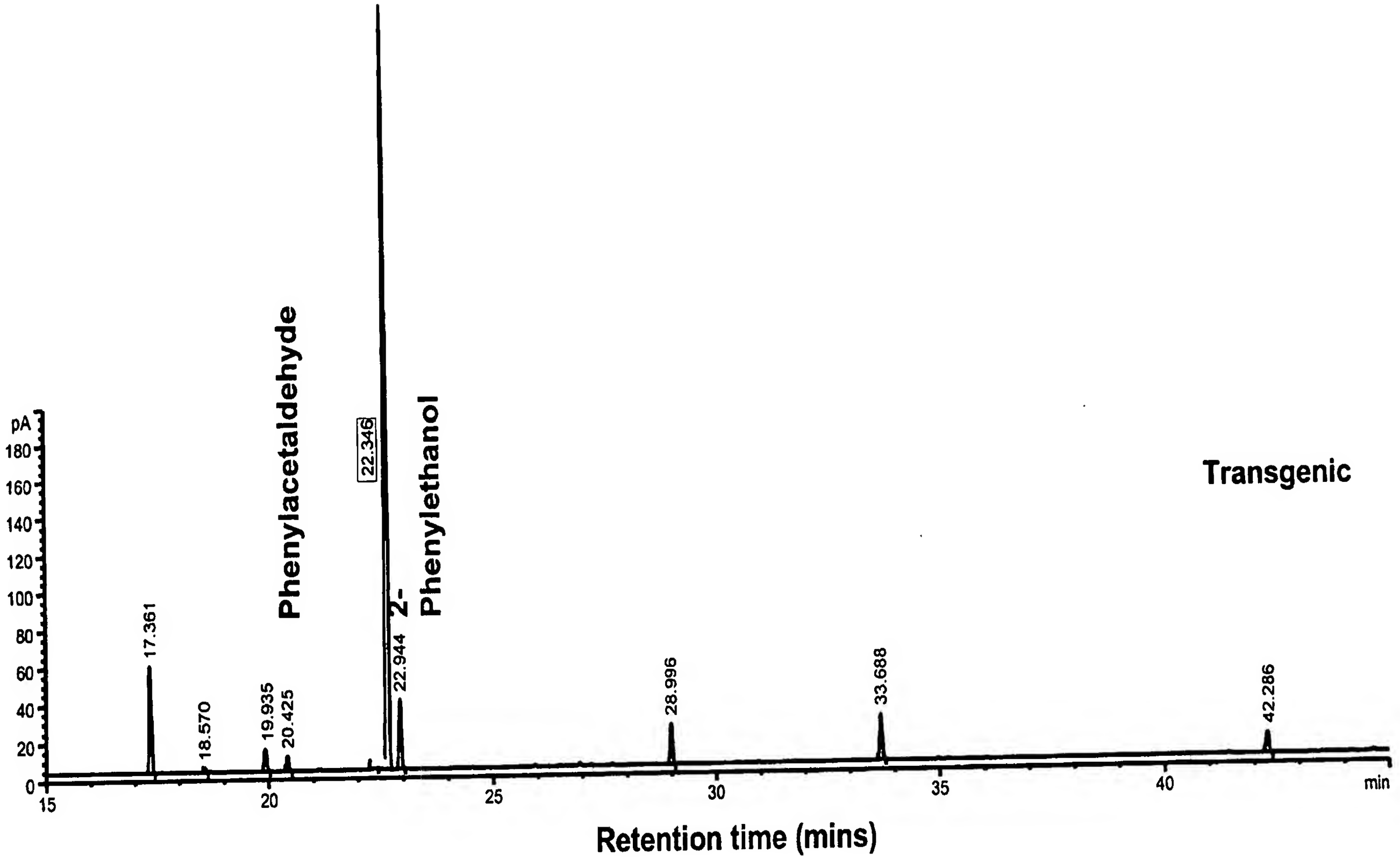


FIG. 5C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19.935
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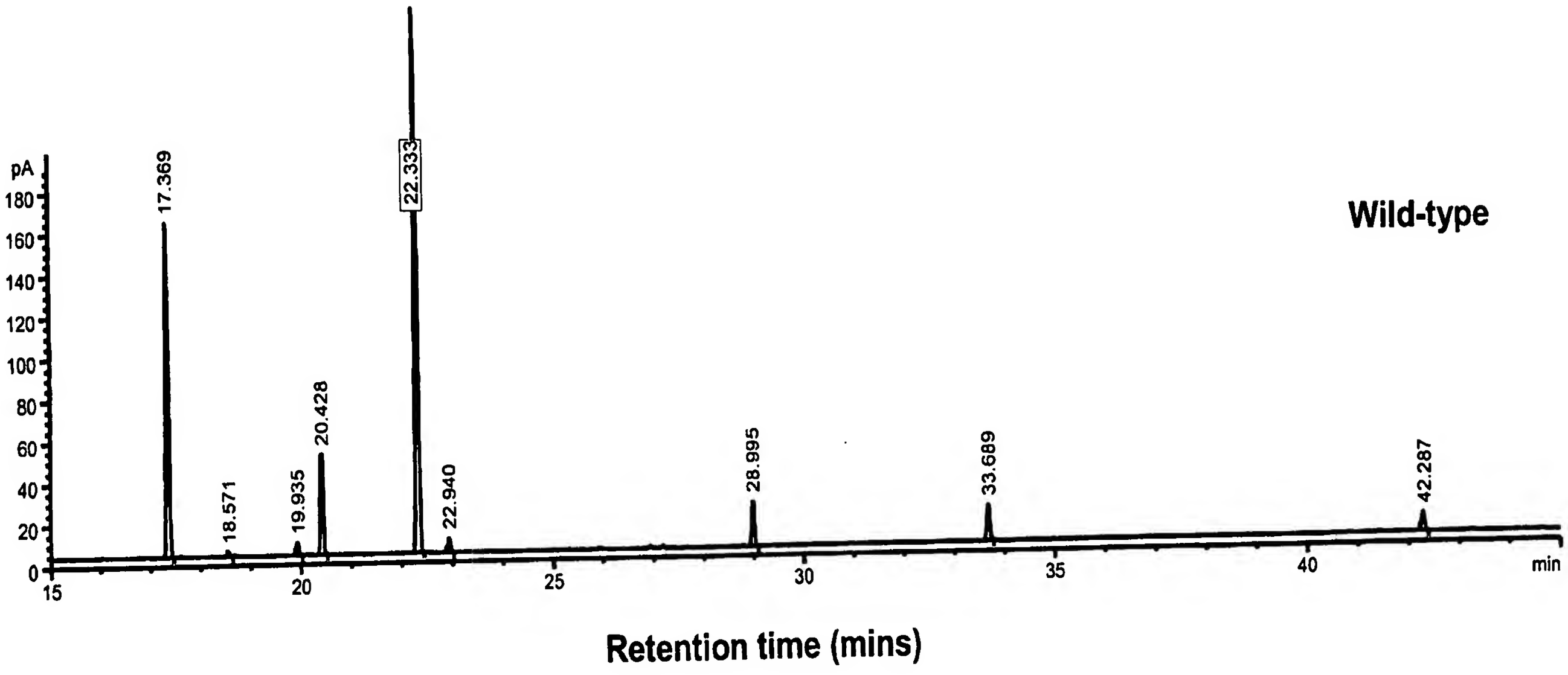
35
40
min
Retention time (mins)

FIG. 6A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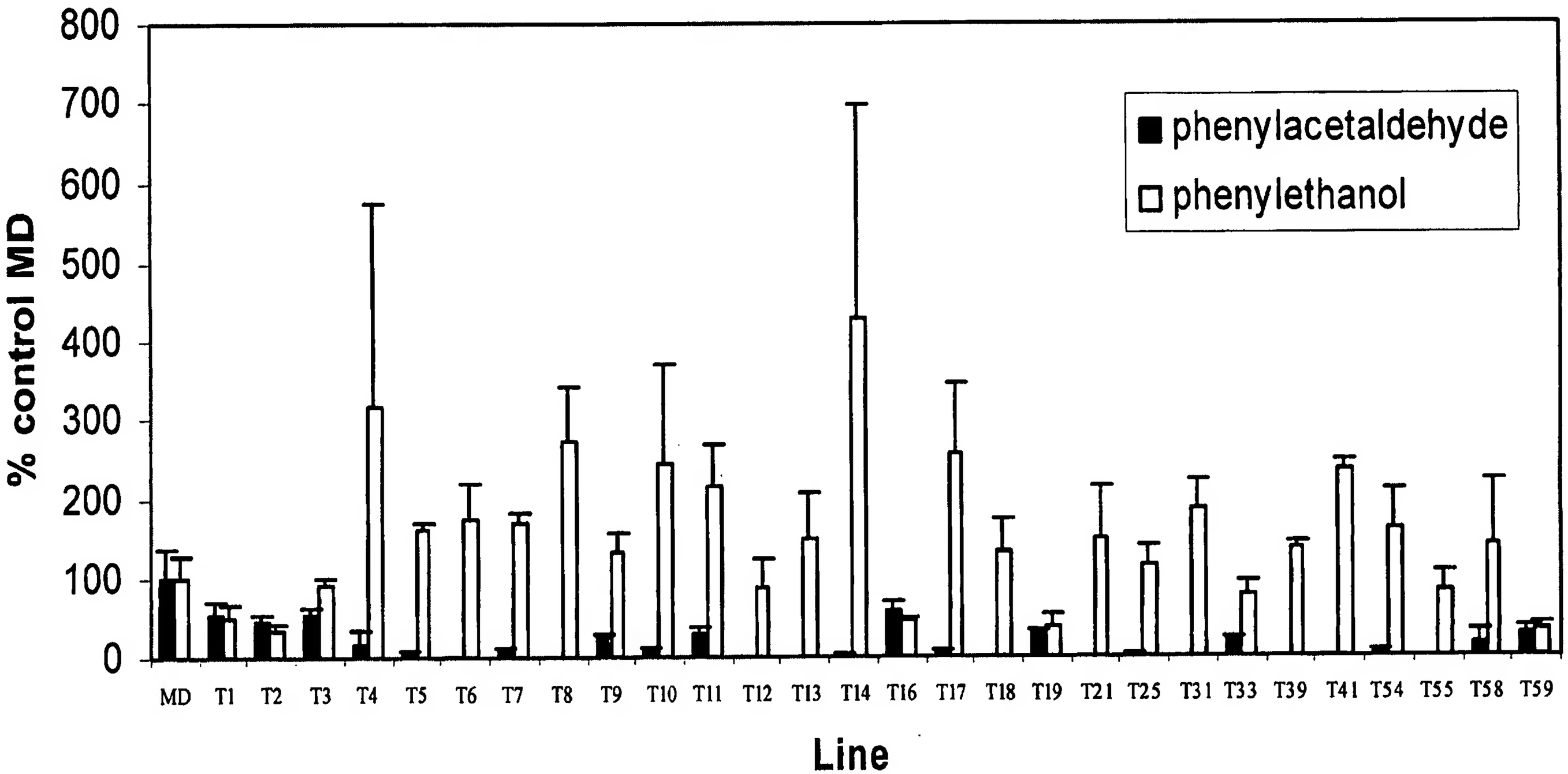
30
35
40
min
Retention time (mins)

FIG. 6B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T5
T6
T7
T8
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T10
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T12
T13
T14
T16
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Line

FIG. 7

SEQUENCE LISTING

<110> Klee, Harry J.
Tieman, Denise

<120> Materials and Methods for Synthesis of a Flavor and Aroma Volatile in Plants

<130> UF-386P

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Val Phe His Thr Ala Ser Pro Phe Tyr Tyr Ser Val Thr Asp Pro Gln
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Glu Asn Pro Ser Ala Asn Gly Arg Tyr Leu Met Val Glu Arg Val Ala
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Gln Leu Pro Glu Lys Cys Ala Asp Asp Asn Pro Leu Met Gln Asn Tyr
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Gln Val Ser Lys Glu Lys Ala Lys Ser Leu Gly Ile Glu Phe Thr Thr
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